# PANEL SESSION 3
# IMMUNOPATHOLOGY

03-01

## DETECTION OF CYTOKINES BY MONOCYTES FROM PATIENTS WITH PARACOCCIDIOIDOMYCOSIS

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Monocytes and macrophages can produce a large repertoire of cytokines and participate in the pathogenesis of granulomatous diseases. The aim of this study was to evaluate the production of pro- and antiinflammatory cytokines in patients with active paracoccidioidomycoses. Monocytes from 37 patients and 29 healthy controls were cultivated with or without 10 microgram/mL of lipopolysaccharide (LPS) during 24h at 37°C, and the cytokine levels were determined in the cultures supernatants by enzyme immunoassay. The results showed that the endogenous levels of TNF-alpha, IL-1beta, Il-6, IL-8, Il-10 and TGF-beta1 detected in supernatant of patients monocytes, cultivated without stimulus, were significant higher than those produced by healthy controls. However, patients monocytes produced significantly lower TNF-alpha and IL-6 levels in response top LPS when compared to normal subjects, suggesting an impairment in the capacity of these cytokine production after LPS stimulation. Concentrations of IL-1beta, IL-8 and IL-10 in cultures stimulated with LPS were higher in patients than in controls. The results demonstrated that monocytes from patientes with active paracoccidioidomycosis produce cytokines with both inflammatory and antiinflammatory activities. The impairment in TNF-alpha and Il-6 synthesis after LPS stimulation suggests that IL-10 and TGF-beta1 may play a role in downregulation of these cytokine production by monocytes. In paracoccidioidomycosis an imbalance production of pro and anti-inflammatory cytokines might be associated with the pathogenesis of the disease.

03-02

## FLOW CYTOMETRIC ANALYSIS OF CELL SURFACE MARKERS IN PATIENTS WITH *Paracoccidioides brasiliensis*

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Paracoccidioidomycosis (PCM) is the most prevalent deep mycosis in Latin America and the record of the cases goes to Brazil. This disease is caused by the inhalation of conidia from the micelial form of the thermo-dimorphic fungus *Paracoccidioides brasiliensis*. Cellular immunity has been the major mechanism of defense against this pathogen while humoral immunity is associated with the severe forms of PCM. In an attempt to establish a phenotypic profile of peripheral blood mononuclear cells (PBMC) from PCM patients presenting different clinical forms, after treatment or not, we incubated these cells with monoclonal antibodies against CD3, CD4, CD8, CD33, CD19, CD28, CD86 and HLA-DR cell surface markers. The flow cytometric analysis of PBMC was performed in an ex vivo and post-culture in the presence of *P. brasiliensis* soluble antigens (PbAg) contexts and preliminary results suggest differences between activated cells before and after culture. We also observed that PbAg induces PBMC proliferation as assayed by blastogenesis.

Supported by CAPES

03-03

## CORRELATION BETWEEN NITRIC OXIDE (NO) PRODUCTION AND TYPE 1/2 CYTOKINES IN IMMUNE RESPONSE AND INFLAMATORY REACTION IN *P. brasiliensis* INFECTED MICE

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PCM is a deep mycosis characterized by suppression of cellular immunity and a suppurative granulomatous inflammation. It is known that nitric oxide (NO) produced mainly by macrophages, besides having microbicidal effects, contributes to the occurrence of the immunosuppression observed during the course of the experimental infection with *P. brasiliensis*. The immussuppression was related to weak and confluent granulomas with immature cells. NO can also influence the outcome of a granulomatous reaction by its effects on the production of cytokines, as observed in others chronic granulomatous diseases. The aim of the present work was to evaluate whether the inhibition of NO caused a differential production of type 1 and type 2 cytokines in mice infected with *P. brasiliensis*. Mice infected with the virulent strain Pb18 were treated with aminoguanidine, which is an inhibitor of the inducible NO synthase, or buffered saline (control). NO production increased significantly in control animals, whereas treated animals had a mild elevation. The immunoproliferative response of spleen cells that had been stimulated with PHA in control animals were depressed and the lungs showed a confluent granuloma. The treatment prevented the impaired proliferative response of spleen cells to the mitogen. In addition, the treatment resulted in well defined and circumscribed granulomas despite the fact that aminoguanidine treated animals had a higher lung fungal burden. Semi-quantitative RT-PCR analysis of the lung revealed that both control and treated animals, after 15 days post-infection, had high expression of the type-2 cytokine IL-10 while IFN-g had a not detected expression. TNF had a higher expression in control animals. At 75 days post-infection there was a substantial increased TNF expression and decreased IL-10 in treated animals. In addition, IFN-g was only detected in treated animals.

03-04

## PRODUCTION OF IL-10, IFN-gamma AND NITRIC OXIDE (NO) BY INTRATHORACIC CELLS EXPOSED TO Paracoccidioides brasiliensis

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Paracoccidioidomycosis is a chronic infection that primarily affects the lungs. Establishment of infection is accompanied by an inflammatory process in which leukocytes are believed to play an important role. In this work we used the intrathoracic route to induce and study the inflammatory response to *Paracoccidioides brasiliensis* (Pb) in the thoracic cavity. BALB/c mice were intrathoracically inoculated with 106 yeast forms of Pb and levels of nitric oxide (NO), IFN-gamma and IL-10 were measured after 48h culture of cells from pleural washes, in the presence of filtrated Pb antigens. The cells were evaluated on the 2nd and 7th days after Pb infection. Lungs from both infected and control mice were collected and prepared for hystopathological studies. On the 2nd day postinfection (p.i.) NO and IFN-gamma production were higher than on the 7th day, in contrast levels of IL-10 only increased at the 7th day p.i. In the hystopathological study was observed the presence of chronic inflammatory infiltrate (lymphocytes and macrophages) associated with a few number of neutrophils. In addition, after 7th days of infection some small perivascular granuloma were detected in the lungs. These preliminary data suggest that early infection by Pb in the intrathoracic model, is accompanied by increased in NO and IFN-gamma production, but by the 7th day IL-10 predominates.

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**03-05**

## EXPERIMENTAL MURINE PARACOCCIDIOIDOMYCOSIS: TH1 AND TH2 PATTERNS AND IMMUNOSSUPPRESSION

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Th1 x Th2 responses of BALB/c mice were studied during *Paracoccidioides brasiliensis* (Pb) infection. Infected and control mice were both divided into two groups, one of which was treated daily with 5mg of azathioprin (AZA), an imunossupressor. Our results show: (i) levels of macrophage activation and IFN-gamma production increased during the first 2 weeks of infection; (ii) exacerbation of the disease correlates to an increased number of CFU of Pb in spleen and fall of IFN-gamma and IgG2a production, in contrast to increased IL-4 and IgG1 after the 60th day postinfection (p.i.); (iii) the daily AZA treatment causes significant impairment of the immune responses of infected mice to all parameters and time periods studied, and mice died earlier with high parasite load in spleens; (iv) when AZA treatment was limited for the first 8 days of Pb infection, macrophage activation and IFN-gamma production increased in infected/treated mice, compared to only infected mice, at the 30$^{th}$ day p.i. In conclusion, Th1 type of responses is predominant during the early phase of Pb infection but it changes to a Th2 phenotype at the late stage. Continuous treatment with AZA depresses both Th1 and Th2 responses and is responsible for most progressive infection and early death of infected mice. However, interruption of AZA treatment induces increased Th1 response in the infected mice.

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**03-06**

## CHARACTERIZATION OF B-1b CELLS AS ANTIGEN PRESENTING CELLS IN THE IMMUNE RESPONSE TO GP43 FROM Paracoccidioides brasiliensis IN VITRO

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Antigen presentation is an essential stage in the development of immune response to a specific antigen. This response can lead to the production of antibodies and/or effector T lymphocyte activation. Macrophages, dendritic cells and B-lymphocytes, among others, act as antigen presenting cells. B-lymphocytes capture antigenic particles through a surface receptor of IgM nature. The interaction IgM-antigen leads to endocytosis of the complex and antigen processing which culminates in presentation of the antigen on the cell surface associated with a class II MHC molecule. At least three B cell subsets, B –1a (Ly-1B), B-1b and B-2, are present in the mouse periphery. B-1a and B1-b cells represent a small population in the adult spleen and are abundant in the peritonial and pleural cavities. It has been demonstrated in our laboratory that B-1b cells spontaneously proliferated in stationary cultures of adherent peritonial cells. Further, that these cells migrate to a non-specific inflammatory focus. Based on these findings, we investigated whether these cells are antigen presenting cells *in vitro* using as antigenic stimulus gp43 from *Paracoccidioides brasilienses*. Results showed that B1-b cells express constitutively high levels of class II MHC and costimulatory molecules inducing an efficient proliferation of gp43 sensitized T lymphocyte.

03-07

## CYTOKINE INDUCTION BY PARACOCCIN: CORRELATION WITH NITRIC OXIDE PRODUCTION

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We have previously demonstrated that paracoccin, a GlcNAc binding protein from *P. brasiliensis* yeast, induces murine macrophages to produce high levels of nitric oxide (NO). In the present work we focused on the paracoccin effect on cytokine production by adherent cells obtained from the peritoneal cavity of C57Bl/6 mice, pretreated (elicited cells) or not (resident cells) with thioglycollate i.p. injection. The *in vitro* stimulus of resident or elicited cells with paracoccin (2.5 mg/ml) persistently induced TNF-a production from 12 to 72 hours after stimulation. Resident, but not elicited cells, produce high concentrations of IFN-g, attaining peaks 12 and 48 h after paracoccin stimulus. Time course curve of NO production by resident and elicited cells was also determined. Concerning resident cells, the NO production profile was correlated to the pattern of IFN-g releasing. There were two peaks of NO production, detected 24 and 72 h after paracoccin stimulus, succeding in 12 and 24 h the first and second peak of IFN-g release, respectively. A decay of NO production, 36 hours after paracoccin stimulus, was associated to a drastic decreasing of IFN-g production. Elicited cells produced increased concentrations of NO, drawing a time-course curve that correlates, with a 12 hours delay, to that of TNF-a releasing by the same cells. The NO production by both, resident and elicited cells, was maximum 72 hours after paracoccin stimulation. Our results suggest that a synergistic action of host (IFN-g and TNF-a) and fungus (paracoccin) factors is accounted for the levels of NO production in *P. brasiliensis* infection. Further investigations are needed to better determine the role of paracoccin as a coresponsible factor for the T cell immunosupression associated to susceptibility to *P. brasiliensis* infection.

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03-08

## DETECTION OF IgE ANTI-CELL FREE ANTIGEN FRACTION OF Paracoccidioides brasiliensis AND IgG-IgE IMMUNE COMPLEX IN THE SERUM OF PATIENTS WITH PARACOCCIDIOIDOMYCOSIS

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In paracoccidioidomycosis (PCM) it is observed an increase of total IgE and IgE anti-gp43 serum level. The present study had the purpose of analyzing the seric level of IgE anti a high mass molecular glicoprotein from *Paracoccidioides brasiliensis* and IgG-IgE immune complex. ELISA plates were coated with goat immunoglobulin fraction anti-human IgE, followed by treatment with serum samples diluted 1:40; CFA antigen fraction (first cromatography peak in Sephadex G-200 column); anti-*P. brasiliensis* antigens IgG (purified IgG from PCM patients serum) and goat anti-human IgG conjugated with peroxidase. For IgG-IgE immune complex detection the same procedure was used, withdrawing the two components used prior to the conjugate. The results obtained from the analysis of 35 PCM patients sera and 17 normal human sera (NHS) were: 0.445 ± 0.111 (PCM) and 0.398 ± 0.061 (NHS), $p > 0.05$, for IgE anti a high mass molecular glicoprotein from *P. brasiliensis* and 0.536 ± 0.186 (PCM) and 0.439 ± 0.082 (NHS), $p<0.05$, for IgG-IgE immune complex. These preliminary results suggest that the formation of IgG-IgE immune complex occurs in PCM.

**03-09**

## IL-10 BUT NOT TGF-BETA INHIBITS *Paracoccidioides brasiliensis* KILLING BY HUMAN ACTIVATED MONOCYTES

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The role of supressor cytokines on the activation process of phagocytic cells to kill *Paracoccidioides brasiliensis* is not yet understood. The propose of this work was to study the role of IL-10 and TGF-beta on the fungicidal activity of normal human monocytes preactivated with IFN-gamma or TNF-alpha and challenged with virulent strain of *P. brasiliensis*. Peripheral blood monocytes (2 $x10^6$/mL) from 12 normal subjects were preincubated with IFN-gamma (1000 U/mL) or TNF-alpha (250 U/mL), in the absence or presence of different concentrations of IL-10 (50, 100 or 200 U/mL) or TGF-beta (5.0, 2.5 or 1.25 ng/mL) for 18h. After, the cells were challenged with *P. brasiliensis* strain 18 (4 x $10^4$ yeast/mL) by 4 h and fungicidal activity was evaluated by plating of the cocultures and counting of colony forming units. The results showed that IL-10 alone or incubated simultaneous with IFN-gamma didn't affect the fungicidal activity. However, the concomitant incubation of TNF-a and IL-10 in an adequate concentration, inhibits this activity demonstrating the capacity of this cytokine to deactivate human monocytes activated with TNF-alpha, with consequent inhibition of killing process. Differently from IL-10, TGF-beta, in adequate concentrations, alone or in a simultaneous incubation with TNF-a induces the cells to a significative fungicidal activity against *P. brasiliensis*.

**03-10**

## INDUCTION OF PROTECTIVE IMMUNITY IN MICE BY FPLC FRACTIONATED *Paracoccidioides brasiliensis* ANTIGENS

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*Paracoccidioides brasiliensis* causes a chronic granulomatous mycosis prevalent in South America, and cell-mediated immunity represents the principal mode of protection against this fungal infection. We investigated whether immunization using FPLC fractions could elicit protective immunity against *P. brasiliensis*. Animals were immunized by subcutaneous injection of either 10 ug fraction 0, II or III in the presence of 100 ug of Corynebacterium parvum and 1 mg of ($Al(OH)_3$) and challenger with pathogenic Pb 1914. Mice immunized with fraction II presented a specific humoral and cellular immune response with high production of IgG1 and IL-10. We observed a significant decreases ($p<0.001$) CFUs quantity measured in the lung in mice immunized by fractions 0 and II. We did not detect the presence of fungous in the spleen or liver of immunized mice with fraction 0 or II. However, the fraction III was not able to induce protection in mice. In addition, we just observed, by indirect immunofluorescence assay, the presence of surface antigens detect by rabbit sera against fractions 0 and II. These results indicate the relation between surface antigen and protective activity. In the same way, the fractions 0 and II showed in previous works to promote *in vitro* cell proliferation and granuloma formation by mononuclear cells from PCM patients. Take together, these findings suggest that fractions 0 and II can became candidates to development a reliable vaccine against *P. brasiliensis*.

03-11

## THE ROLE OF APOPTOSIS IN THE ANTIGEN-SPECIFIC T-CELL HYPORESPONSIVENESS OF PARACOCCIDIOIDOMYCOSIS PATIENTS

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Paracoccidioidomycosis is a deep endemic mycosis associated with an antigen-specific immunodeficiency. To examine the role of apoptosis in this immunodeficiency, peripheral blood mononuclear cells (PBMC) of patients with paracoccidioidomycosis and controls were stimulated with the main antigen of *Paracoccidioides brasiliensis* (gp43) and an unrelated fungal antigen (from *Candida albicans*, CMA) and analyzed for Annexin V and propidium iodide staining by flow cytometry. Controls' PBMC proliferated well to both antigens. Patients' PBMC proliferated only to CMA, but presented higher levels of apoptosis with gp43 and CMA than in their own non-stimulated cultures. Moreover, gp43-triggered apoptosis in controls' PBMC were lower than in patients. Thus, patients' but not controls' gp43-stimulated T cells apparently remained anergyzed and subsequently underwent apoptosis. While CMA-induced apoptosis is likely triggered by activation-induced cell death, this is apparently not the case in gp43-induced apoptosis because of the lack of cell cycling and IL-2 in the gp43-stimulated cultures. However, higher IL-10 levels were found in gp43-stimulated patients' PBMC cultures. Addition of a neutralizing anti-IL-10 antibody to the cultures resulted in increased apoptosis levels only in gp43-stimulated patients' PBMC cultures. Our results suggest that apoptosis play a role in the patients' antigen-specific immunodeficiency and that IL-10 may have an anti-apoptotic role.

03-12

## DENDRITIC CELLS AND PATTERN OF CYTOKINES IN Paracoccidioidomycosis SKIN LESIONS

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We quantified by immunohistochemistry FactorXIIIa+ dermal dendrocytes (DD), Langerhans cells (LC),TNF-alfa,IFN-gama,IL5 and IL10 expressing cells in PCM skin lesions. Biopsies were classified according to the tissue response in well-organized granuloma (G1), poorly-organized granuloma (G2) and samples with both kinds of granuloma (G3). The internalization of *P. brasiliensis* by FXIIIa+DD was visualized by a double immunostaining. G1 had high numbers of IFN-gama+ cells and G2 high number of IL5 and IL10+cells. High numbers of FXIIIa+DD were found mainly in G3, some cells around the granuloma. LC presented short and irregular dendrites. G3 had high numbers of IL5,IL10 and IFN-gama +cells. It showed high number of TNF-alfa+ cells,some of them were dendritic and localized around the granuloma,similar to the FXIIIa+DD localization. We correlated these findings with the probable role of FXIIIa+DD as APC in PCM skin lesions. They could be stimulated by fungal antigens, to produce TNF-alfa and help in the granuloma formation.

**03-13**

## IMPORTANCE OF GP70 IN EXPERIMENTAL PARACOCCIDIOIDOMYCOSIS

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Paracoccidioidomycosis (PCM) is a deep systemic granulomatous mycosis whose ethiological agent is *Paracoccidioides brasiliensis*. The fungus presents a complex protein structure, associated with glycoproteins with molecular weights ranging from 13 to 148 kDa. A cell surface glycoprotein with a molecular weight of 43 kDa is considered the main antigenic component of *P. brasiliensis*, since it is detected in 100% of PCM patient's sera. Another antigenic glycoprotein expressed by the fungus, gp70, is recognized by 96% of sera from PCM patients. The predominant components of this molecule are polyssacharides. Gp70 also induces lymphoproliferative response when tested against lymphocytes from PCM patients. In the present study, we produced monoclonal antibodies against gp70 in order to isolate the molecule from total fungus extracts and investigated the possible role of gp70 on the pathogenesis of PCM. Using the antibody, it was observed by confocal microscopy that gp70 is located in the intracellular compartment of the fungus, even though it was also detected in the culture supernatant. Based on these observations, we tested the effect of gp70 on the phagocytic ability of mouse peritoneal macrophages. It was demonstrated that purified gp70 was able to inhibit phagocytosis of zymosan particles by peritoneal macrophages. We also analysed the effect of passive immunization of mice infected with *P. brasiliensis* using the generated MAbs anti-gp70. It was observed, a significant improvement in lung general appearance, which was accompanied by reduction in the number of fungus and granulomas in the organ, thus suggesting a protective activity of the molecule.

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**03-14**

## EVIDENCE OF POLYCLONAL ACTIVATION DURING EARLY STAGES OF *Paracoccidioides brasiliensis* INFECTION IN CBA/J AND CBA/NXID MICE

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Polyclonal activation is characterized by hypergammaglobulinemia, proliferation of different populations of lymphocytes and production of nonspecific and autoreactive antibodies. It has been demonstrated that polyclonal activation occurs in humans infected by *P. brasiliensis*. In experimental PCM this kind of persistent and usually unspecific activation has been demonstrated by progressive infection, high production of specific antibodies and low DTH responses in susceptible mice at late stages of the disease. One of the lymphocyte subpopulation involved in this activation is the B1 cell subpopulation. We investigated the role of B1 cells at the early phase of PCM. CBA/J and CBA/Nxid (B-1 cells deficient) mice were infected intraperitoneally with 106 yeast forms of *P. brasiliensis* strain 18. Splenocytes from control and infected mice were collected after 5, 10 and 15 days of infection and IgM and IgA secreting cells numbers evaluated by ESA. The production of IgM was increased already after 5 days of infection in CBA/J mice and remain constant until day 15. Otherwise, CBA/Nxid mice did not show variation in IgM levels, compared with control counterparts. The same pattern was observed when IgA secreting cells were measured. CBA/J mice presented almost a two fold increase at the 5th day of infection compared to control mice. The numbers of secreting cells stayed elevated throughout the 10 and 15th days after infection. These results suggest that B1 cells play a significant role in the polyclonal activation induced by *P. brasiliensis* at the early stage of infection.

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**03-15**

## FUNGISTATIC AND FUNGICIDAL ACTIVITY OF HUMAN PMN ACTIVATED WITH IFN-gamma, TNF-alpha or GM-CSF. ROLE FOR $H_2O_2$ AND SUPEROXIDE ANION

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Fungicidal and fungistatic activity of human polymorphonuclear leukocytes (PMN) against high virulent strain of *Paracoccidioides brasiliensis* were studied. These effects were tested in short term (4h) and long term cocultures (24h and 48h). Nonactivated PMN failed to exhibit antifungal activities in all studied periods. However if these cells were IFN-g activated they presented fungicidal effect at 4h of cocultures. However no activity, fungistatic or fungicidal, were detected in further times. TNF-a activated PMN exhibit fungicidal activity at 4h and 24 h, while GM-CSF stimulated PMN to fungicidal and fungistatic activities in short and long terms cocultures. The antifungal effects of IFN-g and TNF-a were inhibited or abrogated when *P. brasiliensis* challenge occurred in presence of catalase (CAT) and superoxide dismutase (SOD) showing the role of $H_2O_2$ and superoxide anion in antifungal activities. For GM-CSF activated cells superoxide anion was the main metabolite involved. Based on these data the role of PMN cells in early and later stages of *P. brasiliensis* is discussed.

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**03-16**

## EFFICIENCY OF DIFFERENT ANTIGEN PRESENTING CELL POPULATIONS IN *Paracoccidioides brasiliensis* INFECTION

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The polar clinical forms of paracoccidioidomycosis are associated with different patterns of immune response: cellular immunity decays in severe cases and is maintained in mild cases. The role of antigen-presenting cells (APC)s on the definition of susceptible or resistant phenotype in paracoccidioidomycosis was studied in an experimental murine model that mimics human disease. Although APCs from both mouse strains were competent to present fungal antigen to functional T cells, these responses were preserved in resistant but lost in susceptible mice during infection. Here we identify functional differences between purified APCs from susceptible and resistant mice using T cell proliferation assays. Macrophages and B-lymphocytes from non-infected resistant and susceptible mice were able to efficiently present fungal antigen to T cells and in both mouse strains, macrophages were more efficient than B-lymphocytes. When comparing the performance of each APC population, both macrophages and B-lymphocytes from resistant mice were more efficient than the respective cells originated from susceptible mice. At 8 weeks of infection, both APC populations preserved their ability in presenting antigen, and in both mouse strains, B-lymphocytes were more competent than macrophages. At 12 weeks of infection, however, only macrophages from resistant mice maintained their ability to efficiently present antigen; macrophages from susceptible mice as well as B-lymphocytes from both mouse strains become incompetent. These results suggest that in the resistant mouse strain, preservation of functional integrity of macrophages as APCs in the later stages of the disease is essential for protective cellular immunity and for the favorable evolution of paracoccidioidomycosis.

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**03-17**

## INHIBITION OF UNPRIMED HUMAN MONOCYTES OXIDATIVE BURST BY HIGH-VIRULENT STRAIN OF Paracoccidioides brasiliensis: THE ROLE OF PROSTAGLANDINS

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*Paracoccidioides brasiliensis*, the etiological agent of Paracoccidioidomycosis survives within non-activated monocytes/macrophages. However TNF-a- or IFN-g plus TNF-a-activated cells exhibit an effective killing against high-virulent strain of this fungus, through a mechanism dependent on $H_2O_2$ release. Then, the purpose of this work was to test if *P. brasiliensis* evolved strategies to evade or disarm the phagocyte effector functions. Unprimed human monocytes were preincubated with *P. brasiliensis* strain 18 during 4h and further evaluated for $H_2O_2$ release. A significative reduction in metabolite release was detected. However, high levels of $H_2O_2$, accompanied of significative killing, were exhibited by monocytes treated with indomethacin, before the *P. brasiliensis* challenge. These studies provide evidence that ingestion of *P. brasiliensis* yeast cells seems to avoid their destruction mediated by the monocytes oxidative defence system through a mechanism of inducing prostaglandins release.

Supported by FAPESP

**03-18**

## KILLING OF HIGH VIRULENT STRAIN OF Paracoccidioides brasiliensis BY HUMAN MONOCYTES: THE ROLE OF OXYGEN INTERMEDIATES

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Fungicidal activity of human monocytes against yeast cells from high-virulent strain of *Paracoccidioides brasiliensis* was studied. Killing was assessed by calculating recovery of viable fungi percentage after cells preactivation during 18h with IFN-g or TNF-a or TNF-a plus IFN-g, challenge by 4h with *P. brasiliensis* strain 18, with further plating of cocultures. Cells preactivation with IFN-g alone did not result in a significant killing. This activity was only achieved after preactivation with TNF-a or TNF-a plus IFN-g. Moreover, killing by these activated cells was not significantly inhibited in the presence of superoxide-dismutase (SOD – a scavenger of superoxide anion) or NG-monomethyl-L-arginine (NG-MMLA - nitric oxide inhibitor), but it was almost totally reverted in the presence of catalase (CAT – scavenger of H2O2). These results strongly suggest a role for H2O2 in the fungicidal activity by TNF-a- or TNF-a plus IFN-g-activated human monocytes against high-virulent strain of *P. brasiliensis*.

Supported by FAPESP

03-19

## ROLE OF GRANULOCYTE-MACROPHAGE COLONY-STIMULATING FACTOR (GM-CSF) ON HUMAN MONOCYTES ACTIVATION IN VITRO FOR HIGH-VIRULENT *Paracoccidioides brasiliensis* KILLING

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GM-CSF is a cytokine produced by activated T-lymphocytes, macrophages, endothelial cells and fibroblasts and it is one of the major hematopoietic growth factors sequenced and cloned up today. This glicoproteic molecule regulates growth and differentiation of both hematopoietic stem cells and blood cells progenitors. Recently, there has been an increasing row for the clinical applications of this cytokine, including infectious diseases and cancer; and many works have showed that, beyond its role on proliferation and differentiation, GM-CSF can also act over mature leukocytes. Hence, starting from the point that GM-CSF can influence immune response by activating human mononuclear cells, we were interested in studying its role on human monocytes activation process for high-virulent *Paracoccidioides brasiliensis* (Pb18) killing. Thus, the cells were preincubated with GM-CSF by 18h, in different concentrations, and then challenged with Pb18 by 4h. Following, we assessed fungicidal activity by plating the co-cultures and counting the Colony Forming Units (CFU) of Pb18 after 10 days. Our results show that GM-CSF is able to activate human monocytes to kill Pb18 yeasts in a dependent-dose manner, comparable to the ability of TNF-a alone or TNF-a plus IFN-g-activated cells.

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03-20

## PRO-INFLAMMATORY CYTOKINE PRODUCTION AND CELLULAR ADHESION MOLECULE EXPRESSION DURING EARLY STAGES OF EXPERIMENTAL PULMONARY PARACOCCIDIOIDOMYCOSIS

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At the inflammatory sites leukocyte recruitment (LR) is mediated through cellular adhesion molecules (CAMs) induced by pro-inflammatory cytokines (pi-CKs). These factores were evaluated during experimental *Paracoccidioides brasiliensis* conidia (*PbC*) infection and their relationship to LR investigated. Male BALB/c mice inoculated i.n. with PBS or $4x10^6$ *PbC* were sacrified at 0 – 4, 7 and 14d postinfection to determine: 1) cellulary in bronchoalveolar lavage (BAL); 2) IL-1beta, IL-6, TNF-alpha MIP-2 in BAL. Lung homogenates and sera; 3) inflammatory response and CAMs (ICAM-1, VCAM-1,CD-18, LFA-1,Mac-1) expression in lungs. Comparisons of infected vs control mice revealed significant leukocyte increase in lungs, highest value at 2d post-infection (4.8 +/- $0.3x10^6$ vs 0.3 +/- $0.3x10^6$ cells)(p less than 0.000001). At 2 and 3d post-infection infiltrated was composed mainly by PMNs (90.8%) and encompassed 40.3% and 41.8% of pulmonary area, respectively. Pi-CKs were observed mainly in the pulmonary compartment (p less than 0.000001) with highest levels during the first 4d, maximal level at 2d 1732 +/- 33; 1230 +/- 9; 660 +/- 4,5; 1306 +/- 8 pg/mL for IL-1beta, IL-6, TNF-alpha and MIP-2, respectively; 4) MACs expression (ICAM-1 and $beta_2$ integrins) increased during the same period, except for VCAM-1 expressed only for the first 2d on vasculara endothelium. LR into the lungs of *PbC*-infected mice is mediated by both pi-CKs (TNF-alpha, IL-1beta, IL-6 and MIP-2) and MACs expression (ICAM-1, VCAM-1, $beta_2$ integrins). This inflammatory process may actively participate in the host-parasite interaction by controlling the initial infection.

**03-21**

## LYSOZYME EXPRESSION IN LUNGS OF MICE INFECTED WITH Paracoccidioides brasiliensis CONIDIA

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*In vitro Paracoccidioides brasiliensis* conidia (*PbC*) are susceptible to nitric oxide (NO) but not to products of oxidative burst released by professional phagocytes. Lysozyme (Ly) produced by these cells is microbicidal and fungicidal attacking cellular membranes or wall components. On fungi the enzyme destroys type 1-3 beta-glucan linkage. We determined Ly expression and NO production in lungs of PbC-infected mice. Male BALB/c mice inoculated i.n. with PBS or $4x10^6$ *PbC* were sacrified at 0 – 4, 7 and 14 d postinfection. The lungs were removed, embebed in paraffin and the inflammatory response (H&E), lysozyme expression (immunohistochemistry) and number of propagules (Alcien blue stain) were determined. Additionally, in a different mouse group, bronchoalveolar lavage (BAL) was done to asses NO production (Griess reaction). For the first 4d postinfection, infected mice showed in their lungs an inflammatory infiltrated conformed mainly by PMNs and macrophages accompanied by strong increase in lysozyme expression. In contrast, NO production was undetectable in BAL. Additionally, a significant decrease (p less than 0.001) in number of *Pb* propagules was recorded at 2d postinfection. It appears that lysozyme participates in the microbicidal mechanism against *PbC*; *in vivo* NO appears not to participate in infection control at least during the early stages of the interaction.

**03-22**

## MODULATORY EFFECT OF INTERLEUKIN-6 (IL-6) ON FUNGICIDAL ACTIVITY OF HUMAN MONOCYTES CHALLENGED WITH HIGH- AND LOW-VIRULENCE STRAINS OF Paracoccidioides brasiliensis

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Monocytes from patients with active paracoccidioidomycosis produce high levels of IL-6 that may play a regulatory role on the activation state of these cells. The objective of the present study was to evaluate the effect of IL-6 on the fungicidal activity of human monocytes infected *in vitro* with high- and low-virulence strains of *P. brasiliensis*. Peripheral blood monocytes obtained from healthy individuals were preincubated with or without IL-6 during 24h. Then, monocyte monolayers were challenged with Pb18 high virulent strain or Pb265 low virulent strain of the fungus by co-culture 4h or 18h. Fungicidal activity of monocytes was assessed by co-cultures plating in BHI-agar and determination of viable fungi recovery. The results demonstrated that the preincubation of monocytes with the Pb18 virulent strain during 4h led to a significant higher fungi recovery when compared to control cultures not stimulated with IL-6. This effect was not observed in co-cultures challenged with Pb18 during 18h and with Pb265 during 4h. However, previous treatment of monocytes with IL-6 enhanced fungicidal activity of monocytes against low virulent Pb265 strain, showed by the lower viable fungi recovery. Together the results suggest that IL-6 may exert a modulatory effect on the fungicidal activity of monocytes against *P. brasiliensis* in a dependence of the fungal virulence and the time of monocyte-*P. brasiliensis* interaction.

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03-23

## INTERFERENCE OF KM+ ADMINISTRATION ON THE PROLIFERATION OF LYMPHOCYTES FROM P. brasiliensis INFECTED MICE

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We have showed that KM+, a mannose-binding lectin from Artocarpus integrifolia, by inducing macrophages to produce IL-12 and inverting the cytokine pattern from Th2 to Th1, exerts a protective effect against L. major infection in BALB/c mice. Since resistance to *P. brasiliensis* infection correlates with efficient Th1 immune response we decided to investigate the application of the lectin KM+ as adjuvant in immunization procedure with *P. brasiliensis* antigens. We injected BALB/c mice with 30 mg of exoantigens from *P. brasiliensis*, accompanied or not by 0.5 mg KM+. The animals of another group were injected with KM+ alone and a control group received PBS. Three days after, the animals were i.v. challenged with *P. brasiliensis* ($10^6$ yeasts of BAT isolate). The response of the animals was periodically monitored during 1 month, by DTH, lung histological analysis and cell proliferation assay. Concerning DTH and lung histopathology, similar patterns of response and lesions were detected among the experimental groups of mice. A striking difference was verified concerning the cell proliferation response to Con-A. As expected, spleen cells from mice of the control group (pre-inoculated with PBS) from the 7th day of infection until the end of observation were unable to proliferate. Mice inoculated with exoantigen alone or KM+ alone presented a proliferative response limited to the first three weeks of infection, on the $30^{th}$ day there was no cell proliferation. On the contrary, mice inoculated with KM+ plus exoantigen presented a proliferative response in all anlysed periods. Our results suggest that the lectin KM+ can positively interferes on the control of *P. brasiliensis* infection, by favouring proliferation of lymphocytes. Further investigations will clarify the whole spectrum of KM+ interference on *P. brasiliensis* infection.

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03-24

## PRODUCTION AND EXPRESSION OF TNF-alpha, iNOS AND NFkB IN PERITONEAL MACROPHAGES AND IN MICE INFECTED WITH Paracoccidioides brasiliensis CONIDIA

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Peritoneal murine macrophages activated with IFN-gamma are fungicidal for *Paracoccidioides brasiliensis (Pb)* conidia through nitric oxide (NO) production. NO-production needs the inducible nitric oxide synthase (iNOS) activity, certain nuclear expression factors (NFkB) and secondary TNF-alpha production. *In vitro* we determined the expression of these factors, as well as of NO production in IFN-gamma activated macrophages. Additionally, TNF-alpha participation in the NO-fungicidal mechanism was investigated. *In vivo* (murine model) and during the early infection stages the following parameters were measured: 1) expression and production of TNF-alpha, 2) NO production and iNOS expression in bronchoalveolar lavages (BALs) and lung homogenates (LHs), and 3) CFUs to asseses fungal clearence in lungs. Results revealed that *in vitro* IFN-gamma activated macrophages had increased NFkB and iNOS expression and high levels of NO resulting in inhibition of conidia-to-yeast (C-Y) transformation, TNF-alpha was absent. Contrarywise, activation with TNF-*a* did not result in NO production but in inhibition of C-Y transformation. *In vivo*, TNF-alpha and TNF-alpha RNAm expression increased during the first 2d in BALs and LHs, respectively. NO production or iNOS RNAm expression were undetectable. Finally, a significant CFUs decrease occurred on the 2d post-infection. These results suggest that during the early stages of *Pb* infection TNF-alpha exerts an important antifungal activity against *Pb* conidia through a NO-independent pathway.

**03-25**

## CAPACITY OF P. brasiliensis IN CHANGE PHENOTYPIC EXPRESSION IN THE STRESS DURING PARASITE-HOST INTERACTION

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*P. brasiliensis* has an extraordinary capacity for adaptation for environmental changes, probably by sensitives indicators of stress load during interaction with the host. In its natural habitat demonstrates the ability to hide in contrast with the explosive production of blastoconidias into infected tissues. In this works, we demonstrated the sequencial process of HeLa infection during the first 30 minutes p.i.. Four sequences of HeLa culture infected by *P. brasiliensis* were prepared and revelead by the following techniques: 1- Coloration by MG-G; 2-Indirect Immunofluorescence developed with antiserum against intracellular forms of the fungus; 3- FAS technique for observation of cytoskeleton actin polymerization; 4-Observation in Scanning Microscopy of the same periods. Analysis of these sequences allowed new observations about cells interations,as forms resembling "bags of exo and endospores" and multiple host-infection, confirming the incredible capacity of phenotypic expression of *P. brasiliensis*.

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**03-26**

## STABILITY OF CELL WALL ANTIGENS OF Paracoccidioides brasiliensis

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Stability of the Soluble Component of Cell Wall Outer Surface of *P. brasiliensis* (SCCWOS) is reported. Four antigenic preparations were obtained from the isolate Pb 113 cultivated at 36°C in Fava-Netto's agar for 5, 10, 15, and 20 days. Antigenic lots were kept at 4°C for 15 years. Their antigenic stability was evaluated by immunodiffusion assay (ID) against 60 sera of patients with PCM (43 with the chronic form and 17 with the severe form) as well as pool of sera of patients with histoplasmosis and aspergillosis and rabitt anti-*H. capsulatum*, anti-*A. fumigatus*, anti-Pb, and anti-gp43 antisera. High reactivity of the different SCCWOS against sera of PCM and hyperimmune anti-Pb and gp43 sera was detected. No cross-reactivity was seen when the SCCWOS were assayed against heterologous sera and antisera (anti- *H. capsulatum* and *A. fumigatus*). SDS-PAGE analysis showed high complexity of protein fractions of cell wall antigens, with proteins of molecular weight ranging from 20 to 100 kDa. Using immunoblotting, we confirmed the specificity and sensitivity of the SCCWOS against a pool of sera of chronic and acute PCM forms; SCCWOS obtained at the $5^{th}$ and $10^{th}$ days showed high reactivity to the 25, 43, 60, 70, 85, and 100 kDa antigenic fractions. It is important to report that the 43 and 70 kDa protein fractions are secreted constitutively up to the $20^{th}$ day culture. We have concluded that the SCCWOS are stable and show well-preserved antigenic determinants, which are confirmed by the high reactivity against sera of different clinical forms of PCM, anti-Pb and anti-gp43 antisera. Some sera were also evaluated employing metabolic antigens obtained form isolate Pb113 cultivated in NGTA medium and in Negroni's modified medium, and kept at 40°C for 15 years. These antigenic preparations showed high specificity against all samples analyzed.

03-27

## EVIDENCE THAT B1 CELLS EXACERBATE P. brasiliensis PULMONARY INFECTION IN MICE

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Resistance to *P. brasiliensis* (Pb) infection is associated with effective cellular immunity, which culminates in phagocytosis and killing of yeasts by activated macrophages. Several factors are referred to as modulators of phagocytes in fungi infection some of them exogenous, like drugs and microbe products, and others endogenous, such as genetic background, gamma-interferon and interleukin-10 (IL-10). Here, we demonstrated that susceptibility to the virulent isolate Pb18 is not similar when Balb/c and Balb/Xid mice were used. The latter strain is deprived of B-1 cells. In preliminary *in vitro* assays, total adherent peritoneal cells from Balb/c mice phagocytosed less heat-killed yeasts than did Balb/Xid animals. To confirm the presumable downregulatory effect of B1 cells on these results, wild type mice were gamma-irradiated to selectively deplete these lymphocytes. Depletion of B-1 lymphocytes from the peritoneal and pleural cavities was confirmed by flow cytometry analysis. A phagocytic index similar to that obtained with cells from Balb/Xid mice was observed when peritoneal cells from irradiated Balb/c were tested. Finally, to access the *in vivo* evolution of Pb infection in such murine lineages, both strains were intratracheally injected with $1x10^6$ viable yeasts. A month later, pulmonary fungal burden was analyzed by the cell forming units method using tissue homogenates of infected lungs. Results show that the fungal load in Balb/c animals was nearly 100-fold higher when compared to results obtained with the Balb/Xid strain. Taken together, data here presented suggest that murine B1 cells have a non-protective regulatory effect on Pb-infection in mice.

03-28

## ROLE OF IRON ON THE MULTIPLICATION AND SURVIVAL OF Paracoccidioides brasiliensis IN HUMAN MONOCYTES.

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We investigated the role of intracellular iron on the capacity of *Paracoccidioides brasiliensis* yeasts, strain 18 (Pb 18), to multiply and to survive within human monocytes (MO) from healthy individuals. We pretreated MO ($2x10^6$ cells/ml) with two types of drugs (chloroquine and deferoxamine) that interfere with normal iron metabolism in a variety of cell types, including mononuclear phagocytes. After, the cells were challenged with Pb 18 ($2x10^4$ yeasts/ml – ratio fungus:monocyte 1:100). Because of its basic properties, chloroquine (Chlor) has been shown to raise endocytic and lysosomal pH interfering with normal iron metabolism, which is dependent on an acidic environment in endocytic vesicles and lysosomes; while deferoxamine (Def) is an iron chelator. Cultures of non treated monocytes showed high viable fungi recovery. Monocytes treated with Def (35mM) or Chlor (10mM) showed a significant decrease on the viable fungi recovery. The effect of Def was reversed by iron-saturated transferrin (holotransferrin – 6mg/ml), and partially reversed by holotransferrin (3mg/ml), but not by iron-free transferrin (apotransferrin – 3 and 6mg/ml). Chlor, which prevents release of iron from transferrin, also decreased the viable fungi recovery, but this effect wasn't reversed by holotransferrin (which releases iron only in an acidic environment) or apotransferrin. This study demonstrates that Chlor and Def decrease the fungi recovery from monocytes by limiting the availability of iron to the fungus.

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**03-29**

## MODULATION OF MURINE EXPERIMENTAL PARACOCCIDIOIDOMYCOSIS BY CHLOROQUINE: POSSIBLE EFFECT ON THE CELLULAR IRON METABOLISM.

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The mechanisms used by *Paracoccidioides brasiliensis* to survive into macrophages are not clear. Cellular iron metabolism is of critical importance to the growth of several intracellular pathogens whose capacity to multiply in mononuclear phagocytes is dependent upon the availability of intracellular iron. Chloroquine, a weak base, has been shown to raise endocytic and lysosomal pH of eukaryotic cells thereby interfering with normal iron metabolism in a variety of cell types. The objective of this work was to evaluate the role of chloroquine in the modulation of the murine paracoccidioidomycosis. BALB/c male mice were infected by i.v. route, with $10^6$ yeast cells of *Paracoccidioides brasiliensis* strain 18. Some groups of mice were just infected (control group) and others were infected and daily treated with chloroquine (40, 80 and 120mg/kg). The animals were sacrificed at 2, 4, and 8 weeks after infection, and evaluated by the lung fungi recovery by plating and recovery of colony forming units (C.F.U.). At 2 weeks after infection, the group treated with 80 mg/kg of chloroquine showed significant decrease of the fungi recovery when compared to control groups. However, more important results were detected at the period of 8 weeks, when groups treated with 40 and 80 mg/kg showed a significant reduction of the lung fungi recovery. The results showed a modulatory effect of chloroquine on the evolution of the murine experimental paracoccidioidomycosis, reducing the severity of the lung infection. This drug could be involved in the limitation of the iron availability interfering in the fungus multiplication inside macrophages. The possible chloroquine therapeutic effect on paracoccidioidomycosis will be discussed.

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**03-30**

## INTRACELLULAR PARASITISM OF P. brasiliensis INTO LYMPHOCYTES FROM CRONIC PATIENTS WITH PARACOCCIDIOIDOMYCOSE

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In previous studies of lymphocyte transformation test (LTT) by phytohemagglutinin developed with cells from patients with paracoccidioidomycosis it was found that several lymphocyte smears of these lymphocyte which had been cultured in medium with autologous plasma exhibited reduced blastogenesis, considerably reduction in the number of these cells, many expressing altered morphology and deeply stained. In sequencial work it was studied the ultrastructure of lymphocytes from patients with paracoccidioidomycosis and these cells after LTT developed in cultures with autologous or heterologous plasma. Comparing the micrographs showed in this work that demonstrated many alterations in the morphological aspect of lymphocytes from patients with our results, we identified in the lymphocytes from patients studied in the previous work many structures as intracellulares forms of this pathogen . In this work the authors observed by immunofluorescence, immunoperoxidase reactions and phase contrast mycroscopy of lymphocytes and blasts from two patients with PCM and from one control, different forms of *P. brasiliensis* into these cells from both patients. These lymphocytes also present irregular and granular morphology in contrast with normal lymphocytes. Many experiments are being developed now to confirm and complete these results.

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03-31

## TH1–TH2 CYTOKINE EXPRESSION IN THE LUNGS OF BALB/C MICE INFECTED INTRANASALLY WITH Paracoccidioides brasiliensis CONIDIA. RELATION TO THE INDUCIBLE NITRIC OXIDE SYNTHASE

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Lungs from male BALB/c mice infected with *Paracoccidioides brasiliensis* (Pb) viable conidia were homogenized and used to determine cytokine expression by RT-PCR and ELISA at different post-infection times. The cytokine profiles allowed to establish several groups in accordance with the post-challenge time as follows: a) inflammatory period (24 hours–1 week), where predominant expression of pro-inflammatory cytokines (IL-6, IL-1b, TNF-a and GM-CSF) was observed, b) transitional period (1–4 weeks), where a mixed pattern of different kinds of cytokines such as IL-12p40, TNF-a and INF-g were detected, c) granulomatous period (8–16 weeks), where a high expression of iNOS and other Th2 type and pro-inflammatory cytokines (IL-4, TGF-b, IL-10 and TNF-a), were noticed. During this last period, iNOS expression correlated with protein expression as detected by inmmunohistochemistry and by *in vivo* inhibition using aminoguanidine. The a ove results lend support to previous reports by our group, indicating than either the Th1 or the Th2 patterns may be involved in PCM and that iNOS production may act by controlling dissemination and granuloma formation.

03-32

## DISTINCT PATTERNS OF HYPERSENSITIVITY REACTIONS TO GP43 IN MICE RESISTANT AND SUSCEPTIBLE TO PULMONARY INFECTION WITH P. brasiliensis

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Competent cellular immunity is considered the most effective response in the control of progression of both human and experimental paracoccidioidomycosis. This work compared resistant (A/Sn) and susceptible (B10.A) mice intrapulmonarly infected with the highly virulent Pb18 isolate concerning their ability to respond when challenged with *P. brasiliensis* gp43. In three distinct periods of infection, expressive edemas were detected just 0.5h after footpad injection, but not 24h later, suggesting the occurrence of immediate type of hypersensitivity (IH). Naive animals treated with serum from infected ones corroborated this result, once the same reaction was noted when challenge with gp43 was carried out. Besides that, IgG1 seric titers were higher than other IgG sub-isotypes in Pb-infected animals. No differences were seen between murine lineages in these respects. On the other hand, hystological analysis of footpads demonstrated a mixed set of inflammatory infiltrate, rich in both mono and polymorphonuclear cells, specially eosinophils, what resembled a late phase reaction of IH. In this regard, A/Sn mice exhibited a striking increase in the mononuclear percentages as the infection advanced, while in B10.A lineage no increases, but a slight decrease were observed. Finally, when analyzing fungal burden by cell forming units, it was noted that A/Sn competence to control dissemination from lungs coincided with the increasing ability to recruit mononuclear cells in response to gp43. In contrast, in B10.A the opposite pattern was observed, leading to a broad colonization of splenic and hepatic tissues.

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**03-33**

## EVALUATION OF ACUTE INFLAMMATORY RESPONSE IN ANIMALS TREATED WITH NIMESULIDE INFECTED WITH Paracoccidioides brasiliensis STRAINS

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The present study have had as objetive evalue the effect of the treatment with nimesulide, a selective inhibitor of the inducible isoform of the enzime ciclooxigenase (COX-2), on the murine acute inflammatory response to *P. brasiliensis* strains. To this, adults swiss mice were inoculated by intraperitoneal route (ip) with $1x10^6$ yeast cells of the Mg4 and Mg5 strains obtained from patients from endemic region of Maringá-Pr, and with Pb18. The mice received 2mg/Kg of nimesulide, 1x/day, ip or diluent. On 1st, 3th and 6th day pos-infection the animals were sacrificed and the peritoneal cavity evaluated for cellular influx; peritoneals macrophages functional activities, by determination of $H_2O_2$ liberation; determination of colonies forming units (CFU); IL-2, IL-4, IL-10, IL-13, TNF-alfa, IFN-gama cytokines and PGE2 levels. With regarding to the cellular influx to the peritoneal cavity the treatment induced an increase of polymorphonuclear leukocytes in the infected animals with Mg4 and Pb18 strains, whereas the monocuclear leukocytes influx was increased only with Mg4 strain. The results showed significant increase in the $H_2O_2$ spontaneous liberation from animals that were inoculated with Mg5 and Pb18, but not with Mg4 strain. For all the strains were rcoverd viable fungi from peritoneal cavity; and the treatment seems do not affect the number of fungi that remain in the peritoneo. Moreover, the nimesulide induced a significant reduction on the PGE2 liberation for the three strains, and increase of the levels of IL-10 in the infected animals with Mg4 strain and IL-13 and TNF-alfa in the animals infected with Mg5 strain, at least on the first days pos-infection. Our results suggest that the prostaglandins participation on the murine inflammatory response modulation to differents *P. brasiliensis* strains involve differents machanisms of action.

**03-34**

## DOES INDOMETACIN INHIBIT PGE2 PRODUCTION AT THE MURINE PARACOCCIDIOIDOMYCOTIC INFECTION?

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This work have had as goal to evalue the effect of the treatment with two differents doses of indometacin (1mg e 2mg/Kg), on the murine acute inflammatory response to *P. brasiliensis* strains. To this, adults swiss mice were inoculated by intraperitoneal route (ip) with $1x10^6$ yeast cells of the Mg4 and Mg5 strains obtained from patients from endemic region of Maringá-Pr, and with Pb18. The mice were treated with indometacin, 1mg or 2mg/Kg or diluent, 1x/day, ip. On 1st, 3th and 6th day pos-infection the animals were sacrificed and the peritoneal cavity evaluated for cellular influx; peritoneals macrophages functional activities, by determination of $H_2O_2$ liberation; determination of colonies forming units (CFU) and PGE2 levels. The results showed that two doses of indometacin has similar effects, both of them increasing discreetly the mononuclear leukocytes influx in the animals infected with Mg4 and Mg5 strains; however, with Pb18 occurred a discreet increase of polymorphonuclear leukocytes only indometacin dose of 1 mg/Kg. The evaluation of the treatment with indometacin indicated significant increase in $H_2O_2$ spontaneous liberation from animals inoculated with Mg4 and Mg5 isolates treated with indometacin, 1mg/Kg; whereas, when the mice were treated with the dose of 2mg/Kg this increase occurred only on the 1st day pos-infection. In mice infected with Pb18 only the dose of 2mg/Kg induced increase on the $H_2O_2$ liberation, at the 1st day pos-infection. The treatment with 1mg/Kg induced significant reduction at the concentration of the PGE2, for all strains; whereas the treatment with 2mg/Kg induced a gradual increase of the PGE2 levels when mice were inoculated with Mg5 strain, but had no influence on PGE2 concentration with Mg4 and Pb18 isolates. On the contrary was expect, occurred an increase at the PGE2 liberation, suggesting that the presence of this isolates seems have induced differents mechanisms of liberation of this mediator, when the animals were treated with higher doses of indometacin. New investigations are needs to explain this question.

03-35

## DETECTION OF TNF-ALPHA, TGF-BETA1 AND IL-10 IN BLOOD MONOCYTES AND IN MUCOCUTANEOUS LESIONS OF PATIENTS WITH PARACOCCIDIOIDOMYCOSIS EVALUATED BEFORE AND AFTER TREATMENT

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Cytokines involved in granuloma formation, tissue repair and macrophage-suppressing activity as TNF-alpha, TGF-beta1 and IL-10 were studied in the skin and mucosa lesions and in supernatants of monocyte cultures from patients with paracoccidioidomycosis evaluated before and 20 days after treatment with sulfametazol-trimetroprin. Monocytes from 24 patients and 25 healthy controls were cultivated with or without 10µg/mL of lipopolysaccharide (LPS) during 24h at 37°C, and TNF-alpha, TGF-beta1 and IL-10 levels were determined by ELISA. The expression of these cytokines was detected using immunohistochemistry in paraffin-fixed biopsies. Monocytes from patients with paracoccidioidomycosis, evaluated before treatment, exhibited high spontaneous levels of TNF-alpha, TGF-beta1 and IL-10. After treatment it was observed diminution of the endogenous levels of TNF-alpha, but with persistence of the lesser capacity of this cytokine release after LPS stimulation. High levels of IL-10 and TGF-beta1 detected in monocyte culture with LPS suggest no impairment in the capacity of these cytokines production by patient monocytes. Immunoreactive TNF-alpha was detected in all skin and mucosa lesions before treatment, showing diffuse deposits involving granulomas and significant cytoplasm staining in infiltrated mononuclear cells and keratinocytes. After 20 days of treatment, the lesions exhibited reduction in TNF-alpha expression. Immunostaining for TGF-beta1 was observed diffusely in the dermis and usually in the cytoplasm of macrophages and giant cells. An increase in the diffuse TGF-beta1 expression in the fibrosis area was observed 20 days after treatment. Granular deposits of IL-10 were detected in the lesions mainly on infiltrated mononuclear cells around the granulomas, occurring a reduction in IL-10 distribution in all skin and mucosa lesions after treatment. The results suggest that the cytokines studied in skin and mucosa lesions may be generated as part of the host response to infection, but also produce local tissue damage and play a role in the immunopathology of paracoccidioidomycosis.

03-36

## ENHANCED PRODUCTION OF MACROPHAGE INFLAMMATORY PEPTIDE 1$\alpha$ (MIP-1$\alpha$) BY ALVEOLAR MACROPHAGES FROM PATIENTS WITH PULMONARY PARACOCCIDIOIDOMYCOSIS

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To investigate the local immune response we analyzed the cellular infiltrate and patterns of cytokine production in bronchoalveolar lavage cells and fluid from patients with pulmonary paracoccidioidomycosis (PCM). The group consisted of 19 patients, 16 male and 3 female, with age ranging from 34 to 65 years. The diagnosis was confirmed by demonstration of *P. brasiliensis* in the sputum or bronchoalveolar lavage fluid (BAL), in addition to serological tests. Cytospin preparations from patients with PCM showed an increased number of lymphocytes in BAL, with a predominance of $CD8^+$T cells. The alveolar macrophages (AM) expression of class II, ICAM-1 and B7-2 molecules was significantly higher than in peripheral blood monocytes (PBM). Cultured AM produced higher levels of IFN-gamma, TNF-alpha, IL-6 and MIP-1alpha as compared with cultured PBM. No differences were detected in relation to IL-8, IL-12p40, IL-10 and TGF-beta BAL fluid from PCM patients contained low but significant levels of IL-6, TNF-alpha and MIP-1alpha, and specific antibodies to *Paracoccidioides brasiliensis*, mainly the IgG2 isotype. These findings indicate that the local inflammatory reaction in the lungs of patients with pulmonary paracoccidioidomycosis is mediated by the inflammatory cytokines IFN-gamma, TNF-alpha-6 and by the chemokine MIP-1alpha, which may play an important role mediating the recruitment of lymphocytes and macrophages to the site of infection.

03-37

## MODULATION OF CO-STIMULATORY MOLECULES AT THE EARLY STAGES OF EXPERIMENTAL PARACOCCIDIOIDOMYCOSIS

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Studies using a murine model of PCM demonstrate that the cell mediated immunity directed to Th1 profile of response determines protector immunity against the pathogen. The analysis of molecular events of cell-cell interaction during the early stages of murine host's immune response is being poorly investigated. These early events are determinant to the induction of an adequate immune response against the parasite. In order to evaluate some of those mechanisms, we infected resistant (A/J) and susceptible (BALB/c) mice i.p. with 106 yeast forms of Pb18, and draining and distal lymph node cells were isolated after 5, 10 and 15 days post infection (p.i.). The expression of key activation molecules was measured by flow cytometry. Resistant mice showed an up regulation of the CD80+, CD86+ and CD28+ cells. The expression of these molecules by cells from susceptible mice were either unaltered (CD80+, CD86+) or down regulated (CD28+). T cells CD28+ were also increased on distal lymph nodes from resistant mice. Susceptible mice presented the same profile as observed on draining lymph nodes. All these molecules participate of co-stimulatory signals of lymphocytes and antigen presenting cells during the cognitive stage of immune response, and its variation can be caused by the lymphocyte migration through the lymphatic circulation, or by an expression modulation, provided by cytokines and chemokines. Taken together these results point for the inability of susceptible mice to mount an adequate activation of T lymphocytes at the early stage of experimental PCM.

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03-38

## COULD AN INCREASED APOPTOTIC RATE IN THYMUS BE A MECHANISM OF IMMUNOSSUPPRESSION IN Paracoccidioides brasiliensis-INFECTED MICE?

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Paracoccidioidomycosis, a deep mycosis caused by the dimorphic fungus *P. brasiliensis*, is an endemic disease in Brazil and other Latin American Countries. Many works have shown that immunosuppressive effects induced by systemic mycosis can be related to primary lymphoid organ damage. Previous studies in our laboratory showed that *P. brasiliensis* is able to invade the thymus, which undergone severe atrophy with degeneration of cortical area and loss of cortico-medullary delimitation. In this study we evaluated the relationship between apoptosis and thymic atrophy caused by *P. brasiliensis* in experimentally infected BALB/c mice. It was observed an increase of apoptotic index featured by immunohistochemistry during early stages of infection. Ultrastructurally, it was found an increase of Golgi apparatus that showed full activity as early as 24 hours post-infection. Besides, other cell alterations as mitochondria degeneration and an augment of activated macrophages could be seen. Taken together, these results suggest that thymic alterations may be involved in the immunosuppressive phenomenon frequently observed during the paracoccidioidomycotic infection.

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03-39

## ADJUVANT EFFECTS OF SYNTHETIC OLIGONUCLEOTIDES (ODN) IN THE TH2-TH1 IMMUNOMODULATION IN SUSCEPTIBLE MICE TO PARACOCCIDIOIDOMYCOSIS (PCM)

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Paracoccidioidomycosis (PCM) is characterized by a suppurative granulomatous inflammation; suppression of cellular immunity and high antibody titters. Its etiological agent is the dimorphic fungus *Paracoccidioides brasiliensis* (P.b.). The outcome of the experimental infection depends on several factors such as the host responses, virulence of the infecting agent and type of adjuvant. Correlation between the PCM clinical forms and pattern of immune response has been investigated in human and mouse model. It was established a genetically controlled murine model, where A/Sn and B10-A mice strains are known models of resistance and susceptibility, respectively. It is known that Th1 response confers protection against some pathogens and also against P.b. Recent studies showed that microbial DNA, included P.b. DNA, is considered a stimulator to T helper 1 immune response. The immunostimulatory sequences (ISS) in microbial DNA, defined as CpG motifs were originally identified as hexamers composed by a central CG dinucleotide flanked by two 5´purines e two 3`pyrimidines and in the central dinucleotide pair, cytosine moieties are unmethylated. Previously we showed that these sequences present in P.b. DNA are able of Th2-Th1 immunomodulation in susceptible mice (B10-A) with infection control by IFN-g secretion and predominant IgG2a secretion in immune response. However, which mechanisms and how immunostimulation adjuvant kinetics of this sequences work, stay unclear yet. Our objective was to investigate the immunostimulation kinetics, by means the ability of ODN to modulate the Th2-Th1 immune response in susceptible mice. Our results showed that the best concentration of ODN to modulate this response was 0,5 mg/ml. In addition, liver and spleen were histopatologically analyzed and we observed a decreased fungal load in these organs in the ODN-treated mice when compared with the unstimulated mice. Many studies about immunomodulation kinetics have been reported and our data suggest that synthetic ODN has immunostimulatory proprieties and this effect is concentration dependent.

03-40

## LACK OF REACTIVITY OF SERA FROM PARACOCCIDIOIDOMYCOSIS PATIENTS IN IMMUNODIFFUSION TESTS IS RELATED TO THE PRODUCTION OF LOW-AVIDITY IgG2 ANTIBODIES DIRECTED AGAINST CARBOHYDRATE EPITOPES

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Immunodiffusion (ID) is the most used serological test for diagnosis and treatment follow-up of patients with paracoccidioidomycosis (PCM). The ID test is highly specific (100%) but its sensibility is low (90%), resulting in false-negative tests. The aim of this study was to determine the profile of antibodies in sera from patients with proven PCM presenting negative results in the ID test. We analyzed 46 sera from patients with active PCM (28 with negative ID and 18 with positive ID) with regard to IgG and IgG subclass response to gp43 antigen (treated or not with sodium metaperiodate) by ELISA and immunoblot. The avidity of the IgG was determined by ELISA treated or not with urea 6M. The immunoblot tests showed that both $ID^{neg}$ and $ID^{pos}$ sera recognize predominantly the gp43 fraction of the antigen used in the ID tests. $ID^{neg}$ sera contain low-avidity antibodies, low levels of total IgG and IgG1, and high levels of IgG2 when compared with $ID^{pos}$ sera. The antibodies in $ID^{neg}$ sera were predominantly directed against carbohydrate epitopes, since the treatment with sodium metaperiodate significantly diminished the antibody reactivity. These results showed that the lack of reactivity of sera from paracoccidioidomycoisis patients in ID tests is related to the production of low-avidity IgG2 antibodies directed against carbohydrate epitopes.

**03-41**

## REGULATION OF T HELPER CELL DIFFERENTIATION IN VIVO BY GP43 FROM Paracoccidioides brasiliensis PROVIDED BY DIFFERENT ANTIGEN-PRESENTING CELLS

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The Paracoccidioidomycosis (PCM) infection can evolve to different clinical forms that are associated to various degrees of suppressed cell-mediated immunity. In the murine model, A/Sn and B10.A isogenic strains of mice are known to be resistant and susceptible, respectively, to this fungal infection. Assuming that the effectors immune response is a consequence of the preferential activation of either Th1 or Th2 subsets, in the present work we evaluated the importance of antigen presenting cells (APCs), dendritic cells, macrophages and B cells in the development of the immune response to *Paracoccidiodes brasiliensis*. We have previously demonstrated that, in resistant mice, purified gp43 seems to be preferentially presented by dendritic cells and macrophages stimulating Th1 lymphokines production, whereas in susceptible mice gp43 was distinguishably presented by B cells, which led to stronger activation of Th2 subsets. In the present work we evaluated the capacity of dendritic cells, macrophage and B cells from susceptible and resistant mice in stimulation naïve T cells *in vivo*. The naïve T cell proliferation assay showed that dendritic cells and macrophages induced high level of proliferation when cells from resistant mice were used. However, only dendritic cells from resistant mice stimulated an efficient Th1 lymphokines pattern. Taken together, our results showed that dendritic cells have a highly developed function in the PCM model as specialized APCs for the initiation of T cell-dependent immune responses and inducing Th1 lymphokine.

**03-42**

## RESISTANCE IN HUMAN PARACOCCIDIOIDOMYCOSIS IS ASSOCIATED WITH INCREASED PRODUCTION OF IL-2, IFN-g AND TNF-a. BY PERIPHERAL BLOOD MONONUCLEAR CELLS

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Cellular immune response to *Paracoccidiodes brasiliensis* antigens (PbAg) was evaluated in patients with the juvenile (JF) and adult (AF) forms of paracoccidioidomycosis (PCM) as well as in a group of infected individuals living in the endemic area but without any clinical manifestation of the disease. The immune profile of this group of PCM-infected (PI) individuals was characterized by: 1) a positive skin test to *P. brasiliensis* antigen, 2) absence of specific antibodies, 3) a vigorous lymphoproliferative response to PbAg, and 4) a typical Th1 pattern of cytokines, with production of TNF-a, IL-2, IFN-g and basal levels of IL-4, IL-5 and IL-10. On the opposite, JF patients showed as impaired proliferative response to PbAg and a cytokine pattern characteristically Th2, i.e., lower TNF-a, IL-2 and IFN- g secretion and significantly higher levels of IL-4, IL-5 and IL-10. These profiles are compatible with forms of higher and lower resistance, respectively. Intermediate immune responses were observed in AF patients, whose specific lymphoproliferative response was lower than in the PI group but higher than in the JF patients. Although the secretion of IFN-g and IL-10 did not differ from the JF group, elevated TNF-a levels was observed in AF patients. Since AF patients are able to control fungal dissemination for decades, they can be considered more resistant than JF patients, who manifest the disease soon after infection.

03-43

## DOES INDOMETACIN MODULATE INFLAMMATORY RESPONSE TO P. brasiliensis STRAINS OBTAINED FROM Dasypus novemcinctus?

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The objective the present project was evalue the effect of the treatment with indometacin on the murine acute inflammatory response to *P. brasiliensis* strains from armadillos (PbA2 and PbA4). Adults swiss mice were inoculated by intraperitoneal route (ip) with $1x10^6$ yeast cells of the Pb18, PbA2 and PbA4 strains, and treated with indometacin (1mg or 2mg/Kg, 1x/day, ip) or diluent. On 1st, 3rd and 6th day pos-infection the animals were sacrificed and the peritoneal cavity evaluated for cellular influx; peritoneals macrophages functional activities, by determination of $H_2O_2$ liberation; determination of colonies forming units (CFU) and PGE2 levels. The results demonstrated that the dose of 1mg/Kg indometacin reduced the plymorphonuclear leukocytes influx for Pb18; increased the mononuclear and polymorphonuclear leukocytes influx for PbA2; increased the $H_2O_2$ spontaneous liberation for PbA2 and PbA4 and reduced the concentration of PGE2 for the three strains. However, the 2mg/Kg dose of indometacin did not promote alterations in the cellular influx, in the H2O2 concentration and PGE2 levels induced by fungic infection for any strains. The evaluation of viable fungi recuperation demonstrated gradual decline for all strains and the treatment with two doses of indometacin did not affect the CFU. Analysing the effects of the two differents doses of indometacin for all strains we realized that the treatment with 1mg/Kg have been more effective in the modulation of the several parameters analyzed in the peritoneal cavity and the PbA2 stimulate a different inflammatory response pattern in relation to PbA4 and Pb18.

03-44

## EVALUATION OF THE COX-2 SPECIFIC INHIBITON ON THE MURINE INFLAMMATORY RESPONSE TO P. brasiliensis STRAINS OBTAINED FROM Dasypus novemcintus

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The objective have been to evalue the prostaglandin (PGE2) participation on the acute inflammatory response to three *P. brasiliensis* strains: one isolated from patient (Pb18) and two from armadillos (PbA2 and PbA4). Adults swiss mice were inoculated by intraperitoneal route (ip) with $1x10^6$ yeast cells of the Pb18, PbA2 and PbA4 strains. The mice were treated with nimesulide (2mg/Kg, 1x/day, ip) or diluent. On 1st, 3rd and 6th day pos-infection the animals were sacrificed and the peritoneal cavity evaluated for cellular influx; peritoneals macrophages functional activities, by determination of $H_2O_2$ liberation; determination of colonies forming units (CFU) from peritoneal cavity; PGE2 concentrations and IL-2, IL-4, IL-10, IL-13, TNF-alfa and IFN-gama levels. The treatment with nimesulide increased $H_2O_2$ spontaneous liberation for Pb18 and increased the mononuclear leukocytes influx for PbA4 but have no effect on any parameters for PbA2. The treatment with nimesulide did not affect the IL-4, IL-10, IL-13 and TNF-alfa levels for all strains studied. Moreover, the PGE2 concentration was reduced for basal levels for Pb18 and PbA4, however, for PbA2 the detected levels in the infection was maintained. In the evaluation of CFU was observed gradual reduction in the number of viable fungi present in the peritoneal cavity for all strains. Taken together the results suggest prostaglandin promote modulation on the murine inflammatory response to differents *P. brasiliensis* strains by differents action mechanisms.

**03-45**

## CYTOKINES IN BRONCHOALVEOLAR LAVAGE (BAL) OF PARACOCCIDIOIDOMYCOSIS PATIENTS

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The purpose of this work was to evaluate the levels of IFN-gamma, IL-2, IL-4, IL-10, TNF and TGF-beta on bronchoalveolar lavage (BAL) from paracoccidioidomycosis patients. The results were compared to those from plasma and culture supernatants from PBMC and monocyte obtained from the same patients. The cytokines were evaluated by ELISA. IL-2 levels evaluated in patients BAL, plasma and supernatants of culture cells were lower than respective controls. No differences were detected in relation to IFN- gamma. Differently, IL-4, IL-10, TNF and TGF-beta were higher in patients BAL, plasma and supernatants when compared to respective control cells. The IFN-gamma, IL-2, IL-4, IL-10 and TGF-beta levels in BAL, plasma and culture supernatants of patients were similar. Exception for TNF where the plasma levels were lower in relation to BAL and PBMC culture supernatants. The results suggest that paracoccidioidomycosis patients independently of cellular compartment evaluated, presented high levels of TH2 cytokines when compared to TH1 ones.

**03-46**

## CELLULAR POPULATIONS AND CYTOKINES IN THE CHRONIC MULTIFOCAL FORM OF PARACOCCIDIOIDOMYCOSIS

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We studied in 27 patients with the chronic multifocal form of Pbmycosis, before treatment, the characteristics of the granulomatous response, considering the cellular populations and the presence in situ of Th1 and Th2 cytokines and TGF-b. These results were correlated with the clinical severity, the levels of humoral and cellular immune competence and serum levels of those cytokines. In punch biopsies from skin or mucosal lesions were evaluated: the number and distribution pattern of neutrophils, eosinophils, giant cells, plasma cells, T CD3+ and CD8+ cells, B cells and NK cells (the last three characterized by immunohistochemistry), number of fungi, and detection of IL-4, IFN-g, IL-10, TNF-a and TGF-b by immunohistochemistry. It was measured the serum levels of IL-2, IL-4, IL-10 and TGF-b and the level of specific antibodies anti-Pb by ELISA and accomplished the paracoccidioidin skin test. Neutrophils, eosinophils, plasma cells, B cells, T CD3+ and CD8+ cells and NK cells were present in different proportions and in a singular patterns of distribution in granuloma, with predominance of T CD3+ cells. IL-4 and IFN-g in situ expression demonstrated four immunostaining patterns: IL-4+/INF-g+, IL-4+/INF-$g^-$, IL-$4^-$/INF-g+ and IL-$4^-$/INF-$g^-$. IL-10, TNF-a and TGF-b were detected in several cellular types. In the patients with Pbmycosis, the serum levels of IL-2 were significantly smaller, the TGF-b significantly larger than normal individuals; IL-10 and IL-4 were similar in both group. These results suggest that in the chronic multifocal form of Pbmycosis as a whole, and in its granulomatous inflammatory response is necessary the participation of Th1 and Th2 cytokines and that the high serum and/or tissue levels of TGF-b and IL-10 could contribute to the immune suppression (documented by the low levels of IL-2).

03-47

**Paracoccidioides brasiliensis EXPERIMENTAL INFECTION ALTERS COMPONENTS OF THYMIC EXTRACELULLAR MATRIX**

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*P. brasiliensis*, as well as other microorganisms, produces several alterations in the thymus of experimentally-infected mice. During experimental infection of BALB/c mice we observed reduction in the thymus weight, structural organ disorganization and augment in the apoptotic index of thymocytes. Here, we evaluated the integrity of thymic extracellular matrix fibrillar components from 1 to 14 days after infection with *P. brasiliensis* by using Gomori's Reticulin and Picrossirius-polarization method to assess possible structural changes induced by the fungus. Reticulin fibers presented crescent disruption and loss of its characteristic spider web pattern in the medullar region. The thymic capsule also showed evident disorganization and mostly discontinued. Despite some fibroblastic activity has been noted, neither thymic matrix recovered their usual arrangement until 14 days post-infection nor fibrosis was observed in thymus. Therefore, alterations in thymic extracellular matrix observed during experimental paracoccidioidomycosis may be compromising T lymphocyte maturation.

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03-48

**BONE MARROW CHANGES IN Paracoccidioides brasiliensis-INFECTED MICE**

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Literature has shown bone marrow invasion by *P. brasiliensis* in human paracoccidioidomycosis. However to our knowledge this phenomenon was not reported in experimental disease yet. Here, the involvement of bone marrow in experimental infection of BALB/c mice was investigated by light and electron microscopy. Early histopathological changes included (i) maturation arrest characterized by an increase in immature blood cell precursors, mainly eosinophilic lineage, (ii) intense vascular congestion when compared with the vessels of normal marrow, and (iii) an increased number of megakaryocytes. Normal pattern of marrow was restored by 28 days post-infection. No lesions such granuloma formation or an abnormal cellular infiltrate was observed. In addition, special stains were unable to detect the fungus. The mechanisms responsible for the alterations described here are still unclear but are probably related more to systemic phenomena affecting host rather than to a direct damage of the marrow blood precursors cells by *P. brasiliensis*.

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