Symposium

MS 1 - IMMUNOMODULATION AND IMMUNOTHERAPY

ANTIBODY RESPONSE AND IMMUNOTHERAPY IN DEEP MYCOSIS: PRELIMINARY EXPERIENCE USING MONOCLONAL ANTIBODIES IN PARACOCCIDIOIDOMYCOSIS.

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Background: Antibody-mediated immunity (AMI) was first described in 1890 by Behring and Kitasato when they observed that the transfer of sera from an immunized to a naïve host protected the latter against the effects of diphtheria and tetanus toxins. The experiments by Behring and Kitasato defined the central procedure that is still being used today for evaluating the efficacy of AMI (reviewed by Casadevall, A.- J. Immunol. Methods 291:1-10, 2004).

After being a central piece in the development of immunology in the early 20th century, the study of the functional aspects of AMI declined in the 1960s. The role of antibodies was taken as completely understood, and with the discovery of T cells, there was increased interest on cell-mediated immunity, which with the rediscovery of innate immunity, steered immunological research away from AMI. By the late 1980s, however, the development of monoclonal antibody (mAb) technology, the discovery of Fc receptors, and generation of mice with defined genetic deficiencies made possible studies that rekindled the interest on the basic mechanisms of AMI (reviewed by Casadevall and Pirofski. Infect. Immun. 72: 6191-6196, 2004).

The relationship between antigen-specific immunoglobulin concentration and protection remains poorly understood for the majority of pathogens. There is conclusive evidence, however, that humoral immunity can modify the course of infection caused by some pathogenic fungi. Immunotherapy studies, especially of cryptococcosis are most advanced. *C. neoformans* has a polysaccharide capsule which is essential for its virulence, and mAbs reactive with GXM can protect against experimental cryptococcosis. The efficacy of these monoclonal antibodies against GXM is associated with several parameters such as isotype, recognized epitope, dose and infective inoculum.

Antibodies in paracoccidioidomycosis (PCM) have been associated to severe disease, whereas protection has always been attributed to cellular immune response. Mattos Grosso et al., (Infect. Immun. 71: 6534-6542, 2003) showed that mAbs to gp70 abolished granuloma formation in the lungs of mice intratracheally infected. In the present work a panel of mAbs directed to peptide epitopes of the gp43 was evaluated *in vivo.*

Methods: Four anti-gp43 IgG2a mAbs (32H, 19G, 17D and 10D) and two IgG2b mAbs (3E and 21F) were analyzed. As a control, an irrelevant mAb (A4) was used. Twenty four hours before the mice were intratracheally infected with $3x10^5$ yeast cells of Pb18, 1 mg of mAb was injected intraperitoneally per animal. Groups of mice were sacrificed 15 and 30 days after the infection and colony forming units (CFU) from the lungs were counted.

Results: Significant reduction in the lung CFUs was observed after 15 days with all mAbs tested with the exception of mAb 32H (IgG2a). However, after 30 days of infection, mAbs 19G and 10D (IgG 2a) and 3E (IgG 2b) were able to control the disease whereas mAbs 32H and 17D (IgG2a) and 21F (IgG2b) caused a significant increase in the number of lung CFUs.

Discussion: Specific antibodies to a major antigen can be protective, nonprotective, or disease enhancing depending on several factors and conditions. We show here that some mAbs to gp43 were able to reduce CFUs from lungs of infected mice whereas others did not. Data suggest that specific antibody responses can have a role in the immune protection against PCM. A better understanding of the parameters that influence AMI can enhance our understanding of vaccine efficacy and host susceptibility to infection.

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COMBINED P10 IMMUNIZATION AND CHEMOTHERAPY IN MICE CHALLENGED INTRATRACHEALLY WITH *PARACOCCIDIOIDES BRASILIENSIS*

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Introduction and Objectives: Previous work has shown that the major diagnostic antigen of *P. brasiliensis*, the gp43, elicits a vigorous IFN-γ-mediated T-CD4+ response that is protective against the intratracheal challenge by virulent yeasts of this fungus. The epitope has been mapped to a 15-aa peptide (P10) that is presented by 3 MHC class II molecules from different mouse haplotypes (Taborda et al., Infect. Immun., 66: 786-793, 1998). The promiscuous nature of P10 was extended to human HLA-DR molecules, as it was shown that this peptide and the analogous gp43 (180-194) without C-terminal asparagine (glycosylation site in the original gp43) and with N-terminal lysine, bound to 9 most prevalent HLA-DR molecules. Furthermore, 4 additional peptides from gp43 were also promiscuous with respect to HLA-DR binding. The gp43 (180-194) was recognized by 53% of patients with treated PCM and the other promiscuous peptides were recognized by 32% to 47% of patients; 74% of patients recognized the combination of 5 promiscuous gp43 peptides (Iwai et al., Molecular Medicine, 9: 209-219, 2003). (for 30 days every 24 h). These results are the basis for devising a peptide vaccine against PCM that could be used as an adjuvant to chemotherapy.

The demonstration that peptide immunization and chemotherapy combined confer increased protection in experimental PCM is a necessary step in this study. Presently, we used P10 immunization along with amphotericin B, ketoconazole, fluconazole, itraconazole, sulphamethoxazole and sulphametoxazole/trimethoprim (5:1) treatment to evaluate the protection against Pb infection in mice.

Methods: Two different protocols were used. After intratracheal infection of male Balb/c mice with 3 x 10^5 yeast cells, drug treatment (for 30 days every 24 h) was started after 48h or after 30 days of i.t. infection. One group of animals was infected and treated with drugs and another was, in addition, also weekly immunized with 20 µg P10, first subcutaneously in complete, and then i.p. in incomplete, Freund´s adjuvant. Mice were sacrificed 30 and 90 days or 60 and 120 days after i.t. infection, and the fungal burden measured as lung, liver and spleen CFUs. Organ homogenates were also assayed for IL-2, IL-4, IL-10, IL-12, TNF-α and IFN-γ using ELISA kits. Lungs were examined for histopathology.

Results: Using Protocol 1, both treatments with drugs and P10 significantly reduced lung CFUs and showed a synergistical effect after 90 days preventing dissemination to liver and spleen. The only relapse obtained with sulphamethoxazole after 90 days was controled by P10 immunization. Using Protocol 2 basically the same results were obtained with itraconazole or amphotericin B plus P10 immunization providing the best protection after 120 days. Using Protocol 1 after 90 days there was a tendency to decreased IL-4 and IL-10 and increased IFN-γ levels in animals immunized with P10. Using Protocol 2 the untreated animal group showed higher levels of IL-4 and IL-10 whereas P10 immunization led to higher IL-12 and IFN-γ levels alone or combined with chemotherapy. The association of drug treatment and P10 immunization rendered large preserved areas in the lungs and fewer granulomas with fewer yeasts cells. In general the association of drug treatment and P10 immunization was highly effective in the protection against intratracheal infection.

Conclusion: Synergistical protection using chemotherapy and P10 immunization supports the adjuvant vaccination of patients with promiscuous peptides to reduce the time of treatment and eventually control anergic cases of paracoccidioidomycosis.

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THE INNATE AND ACQUIRED IMMUNE RESPONSES IN PARACOCCIDIOIDOMYCOSIS: DEFINING THE TARGETS FOR IMMUNOMODULATION AND IMMUNOTHERAPY

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In this talk, the most recent knowledge on innate and acquired immunity of hosts against *Paracoccidioides brasiliensis* (Pb) obtained through the isogenic murine model of resistance and susceptibility to the fungus will be used to develop a rational for immunoprofilaxis or immunomodulation. First it will be shown that a vigorous activation of innate immunity, which is believed to protect hosts from pathogens, does not apply to PCM. A few studies have shown that mannose receptors, complement receptors and laminin are involved in the initial fungal-host cells interactions. Recent work in our lab suggests that Toll Like Receptor-4 (TLR-4), more that TLR-2, functions as a fungal receptor and controls nitric oxide (NO) synthesis but not fungal growth, therefore enhancing Pb infectivity. It has been also demonstrated that NO has multiple antagonistic roles in PCM since this mediator is able to regulate TNF-α synthesis, organization of granulomas, fungal multiplication and suppression of cellular immunity. Unexpectedly, IFN-γ primed alveolar macrophages from susceptible mice are able to control fungal multiplication due to enhanced synthesis of macrophage-activating cytokines and elevated NO production. On the contrary, IFN-γ primed A/J macrophages have a poor fungicidal ability concomitant with negligible amounts of NO due to the enhanced synthesis of TGF-β. Furthermore, differently from expected, a less severe PCM is observed after in vivo inhibition of leukotrienes or in LT-KO hosts. Recent studies on NK cells depletion demonstrated their protective role to normal and T-cell deficient mice; however, in the SCID phenotype NK cells are deleterious. In the same way, PMN leukocytes can be either protective or not, depending on the genetic background of hosts. Altogether, these results firmly indicate that a strong pro-inflammatory response at the innate phase of

immunity does not lead to protective Th1 immunity but appears to activate vigorous regulatory mechanisms which suppress cellular immunity needed to control the disease. Regarding adaptative immunity, several data have shown that the Th1/Th2 model of immunity cannot be unrestrictedly applied to PCM. It is classically accepted that type 1 cytokines are protective whereas type 2 cytokines enhance PCM severity. However, IL-4 which has a deleterious role in the C57BL/6 strain is protective to susceptible mice. PCM in IL-12 KO C57BL/6 mice and in IL-12-depleted B10.A and A/J mice is very severe but early *in vivo* administration of recombinant IL-12 to susceptible mice leads to severe inflammation of lung parenchyma. Moreover, differently from other systemic mycosis, $CD8^+$ T lymphocytes, more than $CD4^+$ T cells exert the main control of Pb growth. Protective immunity in susceptible mice is mainly CD8-mediated, whereas $CD4^+$ T lymphocytes become anergic or deleted due to the intense activation of innate immunity and activation of a $CD8^+$ T-regulatory cell that suppresses cellular immunity. In contrast, in resistant mice the sequential activation of Th1 and Th2 $CD4^+$ T cells and type 1 $CD8^+$ T lymphocytes leads to a regressive disease. Attempts to vaccinate susceptible and resistant mice also brought very interesting results. In B10.A and A/J mice, the previous s.c. inoculation of Pb induces an apparently self-healing infection that can regulate a subsequent challenge. Interestingly, the i.p. and i.v. challenges of s.c. vaccinated mice result respectively in immunoprotection and exacerbated disease. If the challenge is done by the i.t. route immunoprotection is observed in susceptible but not in resistant mice. As a whole, our knowledge on the innate and adaptative mechanisms of immunity to Pb is still fragmentary but reveals the complexity of immune responses, and the major influence of the genetic background of hosts. Our work underscores the notion that activation of innate immunity can inhibit adaptative Th cell responses. As a corollary, a strong Th1 inducing antigen should be avoided to not boost the already hyper-reactivity of some susceptible hosts. Th2-inducing components can lead to exacerbated Th2 inflammatory responses which were shown to be deleterious in some genetic patterns. In addition, individual fungal components or peptides could not stimulate the cooperative action of $CD4^+$ and $CD8^+$ T cells and, more than that, could not stimulate the complex net of interactions which guarantee efficient and sustained protective memory responses.

MS 2 - ECO-EPIDEMIOLOGY AND EVOLUTION OF P. brasiliensis

NEW STRATEGIES AND OPPORTUNITIES FOR THE ECOEPIDEMIOLOGICAL STUDY OF *PARACOCCIDIOIDES BRASILIENSIS*: SENTINEL ANIMAL, MOLECULAR BIOLOGY AND GEOPROCESSING

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The habitat of the saprobe mycelial form of *P. brasiliensis* that produces the infective propagula is yet unknown, since the fungus is difficult to isolate from nature; the disease Paracoccidioidomycosis (PCM) has a prolonged latency period and with no outbreaks. These facts have impeded the adoption of preventive measures to avoid new infections. New opportunities for the study and interpretation of its fungal ecology have emerged with the integrated use of data and information from naturally infected armadillos, molecular techniques that permit fungus detection in soil, spatial statistics and geoprocessing.

Natural infection of *P. brasiliensis* in armadillos (24 *Dasypus novemcinctus*, 03 *Euphractus sexcimctus*, 01 *D. hybridus* and 01 *cabassous tatouay*) was studied in the Botucatu hyperendemic area, including one case from a fluvial island (Serrito Island, Barra Bonita Dam), in which the animals have been geographically isolated from the rest of the continent for more than 40 years. Eight animals (*D. novemcinctus*) were evaluated in captivity for more than two years in relation to PCM development and fungal dissemination to the environment. The fungus detection was carried out by tissue fragment culture, PCR with species-specific primers for *P. brasiliensis* and histopathology. The fungal isolates were characterized for their mycological, molecular and virulence profiles. Soil samples from the environment and armadillo stools were evaluated for fungus presence by animal inoculation and PCR. The area endemic for PCM, including the positive animal sites, was characterized ecologically by its biotic and abiotic factors and with the use of Remote Sensing and Geographic Information System.

Main results: the fungus was isolated in high frequency in the *D. novemcinctus* armadillos; multiple infections can occur in the same animal; humans and armadillos may be infected by the same ecopathogenotypes. Histopathological analysis showed rare granuloma with few fungal elements, suggesting that armadillos can coexist with the pathogen, instead of developing disease. The pathogen was also detected by PCR in the tissues and feces of the animals as well as in soil samples from their burrows. In the species *E. sexcimctus* and *C. tatouay* the fungus was not isolated and/or detected by PCR; but in *D. hybridus* the fungus was detected by PCR in the spleen and mesentheric lymph nodes. Infected animals occur mainly in humid disturbed riparian vegetation.

Spatial statistics demonstrated that the disease does not occur randomly in the study area. Spatial regression among epidemiological data and environmental variables revealed significant correlation between areas with clay soils cultivated by annual annual crops and medium annual precipitation above 1400 mm. *P. brasiliensis* seems to prefer areas that maintain humid conditions most of the time.

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PARACOCCIDIOIDES BRASILIENSIS IN GEOLOGICAL TIMES

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Life on earth started around 4,000 million years ago (Mya), when archaebacteria and anaerobic bacteria appeared, probably derived from a hypothetical Last Universal Common Ancestor (LUCA). Primitive eukaryotes arose 1,500 Mya, whereas fungi made their appearance before the Cambrian explosion took place (600 Mya). Chytridiomycetes, aquatic fungi, are the most primitive within the Kingdom Fungi, followed by Zygomycetes (500 Mya), Basidiomycetes (400 Mya), and Archaeoascomycetes of the type of *Pneumocystis carinii* (320 Mya) or *Schizosaccharomyces pombe* (240 Mya). Euascomycetes evolved in this geological time (e.g., *Neurospora crassa* and *Sporothrix schenkii* 180 Mya), while Hemiascomycetes like *Candida albicans* and *Saccharomyces cerevisiae* made their appearance around 130 and 80 Mya, respectively. As a point for reflexion, *Homo sapiens* has been around only for the past 200 thousand years.

Evolutionary relationships among *Histoplasma capsulatum*, *Blastomyces dermatitidis* and *Paracoccidioides brasiliensis* are under discussion. From protein coding genes, *Paracoccidioides* seems to be the oldest lineage, but *Histoplasma* is the oldest one according to 18S rRNA. Probably all three diverged around the same time. Divergence of *Blastomyces* and *Histoplasma* occurred around 128 Mya, within the Mezosoic Period. Likewise, a mere 12.8 Mya is the probable divergence time for *Coccidioides immitis* and *C. posadasii*.

Setting the probable appearance of *P. brasiliensis* within the late Jurassic and early Cretaceous period, geologically, what is today's South America was close to Africa and separated from current North America. Climate was cold. Could we speculate that *P. brasiliensis* existed only as a saprophyte in the mycelial form? At the beginning of the Tertiary era (Paleocene; 65 Mya) the temperature rose in such a way that if *P. brasiliensis* had already able to shift to a yeast phase, it necessarily had to be forced into this morphology even as a saprophyte. Coincidentally, this time is also the appearance time of the Order Xenarthra (*Glyptodon* and its descendants, armadillos, among them), exclusively in South America, one of whose species is *Dasypus novemcinctus*, nowadays proposed as the main reservoir of *P. brasiliensis* in Nature. Body temperature in armadillos is approximately the same as the median environmental temperature was in the Paleocene. Again, could we hypothesize that by moving into a host, the fungus was able to live in a friendlier environment inasmuch as it provided better and more stable nutritional sources, while enjoying a similar temperature?

During those early geological times, what is now the northern part of South America (e.g., Colombia, Venezuela, northern region of Brazil) enjoyed a tropical, humid climate, whereas current southern Brazil was mainly a desertic, arid region. Once more, could we conjecture if, in addition to possible geographic barriers, climatic differences could drive the currently detected cryptic species in *P. brasiliensis*?

CRYPTIC SPECIATION AND RECOMBINATION IN THE FUNGUS *PARACOCCIDIOIDES BRASILIENSIS* AS REVEALED BY GENE GENEALOGIES

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Introduction and Objectives - *Paracoccidioides brasiliensis* is the etiologic agent of paracoccidioidomycosis, a disease confined to Latin America and of marked importance in the endemic areas due to its frequency and severity. This species is considered to be clonal according to mycological criteria and has been shown to vary in virulence.

Methods - To characterize natural genetic variation and reproductive mode in this fungus, we analyzed *P. brasiliensis* phylogenetically in search of cryptic species and possible recombination using concordance and nondiscordance of gene genealogies with respect to phylogenies of eight regions in five nuclear loci.

Results - Our data indicate that this fungus consists of at least three distinct, previously unrecognized species: S1 (Species 1 with 38 isolates), PS2 (phylogenetic species 2 with six isolates) and PS3 (phylogenetic species 3 with 21 isolates). Genealogies of four of the regions studied strongly supported the PS2 clade, composed of five Brazilian and one Venezuelan isolate. The second clade, PS3, composed solely of 21 Colombian isolates, was strongly supported by the a -tubulin genealogy. The remaining 38 individuals formed S1. Two of the three lineages of *P. brasiliensis*, S1 and PS2, are sympatric across their range, suggesting barriers to gene flow other than geographic isolation.

Conclusion - Our study provides the first evidence for a possible sexual reproduction in *P. brasiliensis* S1, but does not rule it out in the other two species.

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