Experimental pulmonary paracoccidioidomycosis in mice: Morphology and correlation of lesions with humoral and cellular immune response

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Abstract

The present paper describes a murine model for pulmonary paracoccidioidomycosis injecting 6×10^5 yeast forms of *Paracoccidioides brasiliensis* (Pb) by the direct intratracheal route. The sequential histopathology of lung and dissemination lesions together with humoral (immunodiffusion test) and cellular immune response (footpad test and macrophage inhibition factor assay – MIF assay) were investigated since the 1st to the 360th day after infection. All infected animal showed pulmonary Pbmycosis up to Day 30; onwards the lesions subsided being found only in one mouse at Day 360. Dissemination lesions were observed in paratracheal and cervical lymph nodes in 9 out of 68 infected animals. Histologically early lesions were rich in polymorphonuclear cells and evolved to a macrophage desquamative pneumonitis at Day 15 and to typical epithelioid granulomata from Day 30 up to Day 360. Specific precipitating antibodies were first detected 15 days after infection, peaked from Day 30 to 60 and were not observed at Day 360. Significant cell-mediated immunity to Pb was noted at Day 15 with the peak reaction at Day 60 and 90.

The intratracheal route represents a highly effective way of infecting mouse with Pb. This experimental pulmonary Pbmycosis is a granulomatous inflammation which courses with specific humoral and cellular immune response. It may be a good tool for further investigation in the pathogenesis and natural history of the disease.

Introduction

Paracoccidioidomycosis (Pbmycosis) is an endemic deep mycosis frequent in Brazil, particulary in the hinterland of the southern state of São Paulo where Botucatu is located. Therefore several works dealing on the clinical, diagnostic, immunological and pathogenetic aspects of the mycosis have been done at the Botucatu Medical School (6, 11, 15, 16, 35).

One of the most intriguing and debatable feature of the natural history of the mycosis is how and where the *Paracoccidioides brasiliensis* (Pb) penetrates the host and starts the infection (5). At the present, most authors believe that the infection affects primarily the lungs and afterwards may disseminate, particulary to skin and mucous membranes (12, 30). On the other hand, there is experimental (15, 27) and clinical evidence (25, 26, 28) indicating the relevance of humoral and/or cellular immune response in the pathogenesis and evolution of Pbmycosis.

The present paper deals with the establishment of an experimental model of pulmonary Pbmycosis in the mouse with emphasis on the study of the histopathology of the lesions and its correlation with humoral and cellular immune response.

Mycopathologia 77, 3–11 (1982). 0301-486x/82/0771-0003/$1.80.
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Material and methods

Experimental design

Pulmonary Pbmycosis was induced in mice by the direct intratracheal route (i.t.) according to the coccidioidomycosis model as proposed by Lawrence *et al.* (21). Firstly a dye solution was injected i.t. in a group of control mice in order to know the fate and distribution in the lungs of the injected solution. Thereafter a group of mice was infected with a suspension of Pb and the sequential histopathology of the pulmonary and extrapulmonary lesions was studied; the specific antibody production was measured by immunodiffusion test and specific cell-mediated immunity determined by footpad test and macrophage migration inhibition factor assay.

Lung distribution of a methylene blue solution injected by the intratracheal route

Thirty male and female mice with 2 weeks of age were i.t. inoculated with 0.05 ml of a 10% aqueous solution of methylene blue. Briefly the mouse was anesthetized by intraperitoneal injection of sodium pentobarbital; the neck of the animal was hyperextended over the edge of a support; the skin of the anterior neck was excised and the trachea exposed. Then the support was verticalized and an intradermal needle was inserted into the trachea for injection of the dye. Immediately after, the animals were sacrificed; the fate and the gross distribution of the dye in the lungs, trachea,'mouth, esophagus and stomach were then registered.

Experimental infection

Fifty male and fifty female mice with 4 weeks of age were used in this experiment. Sixty-eight mice (infected group) were inoculated i.t. with 0.05 ml of a saline suspension of a 14 day-old yeast culture of Pb* in Fava Netto's culture medium (9). The suspension contained 1.2×10^7 fungi/ml as counted in a hemocytometer chamber. The remaining 32 mice (control group) were similary injected with 0.05 ml of sterile saline. After inoculation, the skin excision was closed and the animals were fed *ad lib* and observed daily. The pulmonary and the extrapulmonary lesions as well as the humoral and cellular immune response of the infected and control mice were studied since Day 1 up to Day 360 (Table 1).

*Strain no. 18 of the Department of Microbiology, Faculdade de Medicina da Universidade de São Paulo, kindly provided by Prof. C. Fava Netto.

Immune assays

Antigen

A soluble antigen was prepared from yeast cultures of strain no. 18 of Pb by sonication (PbAg) (28). The antigen was filtered through a 0.22 μm Millipore filter and submitted to sterility tests. The concentration of protein was determined by the methods of Lowry *et al.* (23).

Footpad test

Twenty-four hours prior to sacrifice, all animals were challenged with a 0.04 ml injection of PbAg (320 μg of protein) into the right-hind footpad; as control the left-hind footpad was injected with 0.04 ml of sterile saline (27). Cell-mediated immunity (CMI) was determined by the increase of footpad thickness measured by pletismography (27). To ascertain the presence and the intensity of the

Table 1. Distribution of the 68 *P. brasiliensis* intratracheally injected mice* and the 32 control animals* according to the interval between inoculation and sacrifice and studies performed: light microscopy (LM); immunodiffusion test (IDT); footpad test (FPT); macrophage inhibition factor (MIF).

Time between inoculation and sacrifice (days)	Number of mice Infected group		Control group	
	LM IDT FPT	MIF	LM IDT FPT	MIF
1	2	–	2	–
3	2	–	2	–
5	2	–	2	–
9	2	–	2	–
15	12	6	6	4
30	12	6	6	4
60	12	6	6	4
90	12	6	6	4
360	12	6	–	–
	Total = 68		Total = 32	

* In each period, it was studied an equal number of male and female mice.

mononuclear cell infiltration, both hind-footpads were removed, fixed in 10% formalin, embedded in paraffin, sectioned and stained with hematoxylin-eosin (HE). The mononuclear cell infiltration was quantified as mild, moderate, intense or severe.

Macrophage migration inhibition factor (MIF) assay

MIF assay was performed by the technique of David *et al.* (8), slightly modified. Immediately after sacrifice, the spleens were removed and placed in a small quantity of cold Eagle's minimal essential medium (Eagle's). The cells were obtained by perfusing the spleens several times with phosphate buffered saline pH 7.2 (PBS) and washed twice with cold PBS. The cells were resuspended in a concentration of 50×10^6 cell/ml in Eagle's. The cell suspension was filled in capillary tubes, sealed at the bottom and centrifuged at 1000 rpm for 10 min at 4 °C. The tube was cut at the cell-liquid interface. The segment containing the cells was placed horizontally in a migration chamber. Then they were filled with Eagle's containing either no antigen (control chamber) or PbAg (test chamber) at a concentration of 300 μg of protein per ml. Preliminary experiments showed that this antigen concentration was able to inhibited the migration of spleen cells 'in vitro' from chronically infected mice without any effect upon migration of spleen cells from normal animals. After sealed, the migration chambers were incubated for 18 h at 27 °C. Areas of migration were measured by using an eye-piece micrometer (Zeiss, no. 474014). All tests were carried out in duplicate. The results were expressed as migration inhibition index (MII):

$$\text{MII} = \left(1 - \frac{\text{mean area of migration with antigen}}{\text{mean area of migration without antigen}}\right) \times 100$$

Immunodiffusion test

Mice were bled via the heart and serum samples stored at −20 °C. Immunodiffusion tests were carried out as previously described (27). All sera were tested in two-fold dilutions and the PbAg was used at a concentration of 3 mg of protein per ml. The results were expressed as the highest serum dilution reciprocal giving precipitation band.

Histopathology

After sacrifice, the cervical and thoracic organs of all mice were removed; for fixation the lungs were injected by the trachea with 10% neutral formalin. After 24 h, the lungs were isolated and paraffin embedded. The cervical organs, including the trachea, esophagus and surrounding soft tissues, were sequentially and transversally sectioned and paraffin embedded. Samples from the tongue, cervical and tracheobroncheal lymph nodes, spleen, kidneys, adrenals, liver and ileal-cecal intestine were also examined. From the paraffin blocks, 5 μm thick sections were cut and stained with HE and silver-methenamine method.

Results

Lung distribution of the methylene blue solution

The methylene blue solution injected i.t. stained both lungs in 90% of the mice and showed an irregular distribution. Most animals regurgitated some of the dye which stained the oral and esophageal mucosa. In a few mice, the dye leaked out from the trachea through the needle orifice and stained the paratracheal tissues.

Experimental infection

The infected mice presented good recovery from the small surgery for the i.t. injection and at no time did they appear in ill health.

Since there was no influence of the sex of the animals on the course of the infection, the immunological and histopathological findings in female and male mice will now be described altogether.

Histopathology

The lesions were considered specific or non-specific according to the presence or absence of Pb, respectively. Table 2 shows the number of mice with specific lesions in each experimental period. All mice developed pulmonary Pbmycosis up to Day 30; onwards the infection subsided, and it was found only in one mouse at Day 360.

Lungs. At Day 1 and Day 3, there were multiple foci of bronchopneumonia around the inoculated fungi.

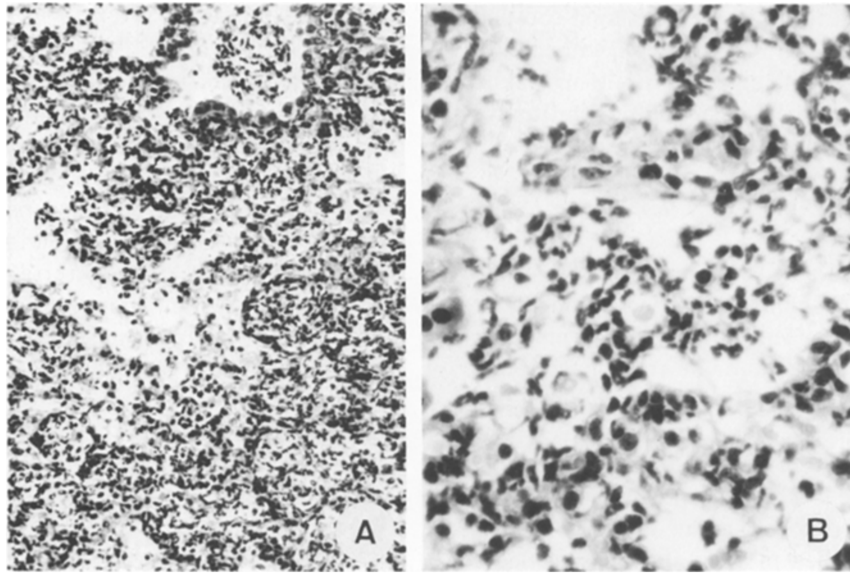

Fig. 1. A. Day 1: Non-specific bronchopneumonia surrounding inoculated fungi. (Hematoxilin & Eosin, 94×). B. Day 3: PMN's encircling inoculated fungi (Hematoxilin & Eosin, 192×).

Table 2. Number of mice with specific lesions (lungs, paratracheal tissues and regional lymph nodes) in each experimental period.

Number of mice with specific Lesion in:	Experimental period									Total
	1	3	5	9	15	30	60	90	360	
Lungs	2	2	2	2	12	12	10	8	1	51
Paratracheal tissues	0	2	1	1	7	5	5	5	0	26
Regional lymph nodes	0	0	0	0	1	3	2	3	0	9
Number of mice studied in each period	2	2	2	2	12	12	12	12	12	68

The inflammatory reaction affected both the alveolar septa and lumina and was mainly by polymorphonuclear neutrophils (PMNs) which frequently crowned the fungi (Fig. 1). The control animals showed foci of intraalveolar PMNs infiltration which were not present after Day 3.

At Day 5 and Day 9, the inflammatory foci were better demarcated, with predominant mononuclear cell infiltration which showed an incipient epithelioid granulomatous organization. The fungi showed multiple exosporulation with a few isolated small forms. At Day 15, there was a change in the pattern of the bronchopneumonia which showed poorly demarcated foci with great number of mononuclears at the alveolar septa and lumina confering to the whole the aspect of a 'desquamative pneumonia' (Fig. 2). In the lesions, there were few fungi, usually in active exosporulation and a small number of lymphocytes and plasma cells at the

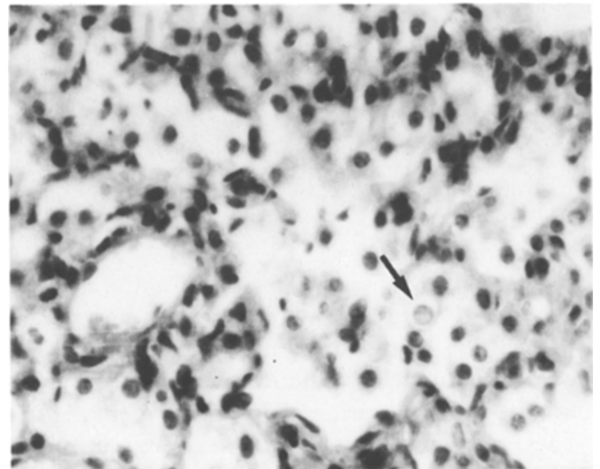

Fig. 2. Day 15: Macrophagic alveolitis around intraalveolar fungi (arrow). (Hematoxilin & Eosin, 480×).

periphery. At Day 30 and Day 60, all the inflammatory foci showed typical well demarcated epithelioid granulomata. There were numerous giant cells and fungi in the lesions. The foci were usually encircled by lymphocytes and plasma cells. Some of the granulomata contained small collections of PMNs (microabscess) (Fig. 3). At Day 90, the inflammatory pattern was qualitatively similar to that of the previous periods; however granulomata were smaller and fungi scarce. Some areas showed incipient fibrosis and foci of calcification. At Day 360, only 1 mouse showed active specific pulmonary lesions, similar to those described at Day 90. The other animals exhibited no pulmonary remaining lesions of the previous infection.

Inoculation site. 38.4% of the infected mice developed mycotic lesion at the paratracheal muscles (Fig. 4a) and interstitial tissues ȧdjacent to the injection site in the trachea (Table 2); the inflammation was granulomatous and contained variable number of fungi in active exosporulation.

Extrapulmonary dissemination of lesions. Dissemination of the infection to the regional lymph nodes was observed in 9 of the 68 infected mice (Table 2). The inflammation showed a granulomatous pattern with few fungi (Fig. 4b). In 5 of those animals, there were specific lesions both in lungs and in lymph nodes; in 3, there were specific lesions in lungs, lymph nodes and paratracheal tissues; in 1, there were only metastatic lesions at lymph nodes.

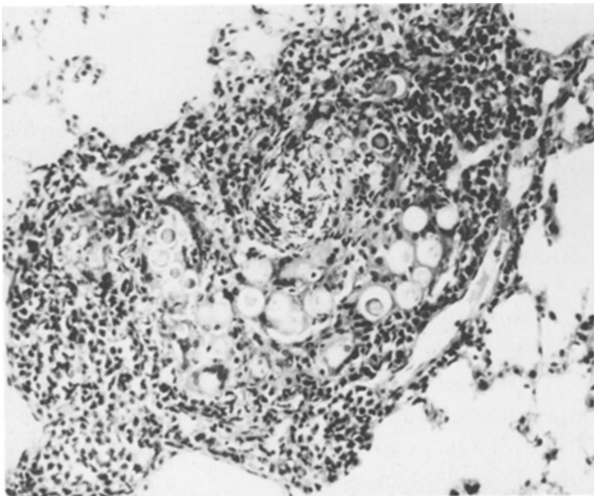

Fig. 3. Day 60: Granulomatous inflammation with epithelioi and giant cells containing numerous fungi in active exosporulation and microabscess. Mononuclear cells are seen surrounding the granulomata. (Hematoxilin & Eosin, 192×).

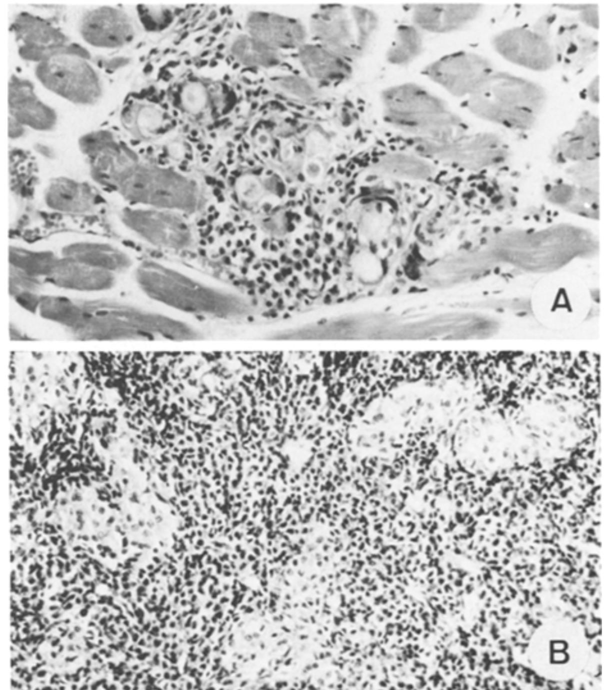

Fig. 4. A. Day 90: Focal myositis at the inoculation site showing mononuclear and giant cells enclosing fungi (Hematoxilin & Eosin, 192x). B. Cervical lymph node showing epithelioid granulomata containing few fungi (Hematoxilin & Eosin, 192×).

Immune assays

Footpad test. As seen in Table 3, the infected mice did not show significant increase of footpad thickness in all periods of study. The histological reading of the tests revealed that there was significant accumulation (intense and severe) of mononuclear cells in the antigen injected footpad in 14 infected mice (20.5%), from Day 15 up to Day 90. Most of those animals (78.6%) were at the 30th and 60th day of infection.

MIF assay. The 5th percentile was used to fix the limit for normal response; migration whose indices were not equal or smaller than 18 were considered as inhibited (Fig. 5). MIF response in the infected animals was present in 3 mice at Day 15 (50%), in 2 at Day 30 (40%), in all animals at Day 60 and 90 and

Table 3. Footpad test: response of cell-mediated immunity with PbAg (320 μg) in infected and control mice measured by pletismography and expressed as increase of footpad thickness (in cm) 24 h after antigen challenge.

Group	Experimental period (days)				
	15	30	60	90	360
Control*	0.620 ± 0.38	0.703 ± 0.25	0.650 ± 0.13	0.572 ± 0.25	Not done
Infected**	0.752 ± 0.27	0.869 ± 0.32	0.676 ± 0.25	0.793 ± 0.25	0.965 ± 0.31

* Arrithmetic mean of 6 animals ± standard deviation.
** Arrithmetic mean of 12 animals ± standard deviation.

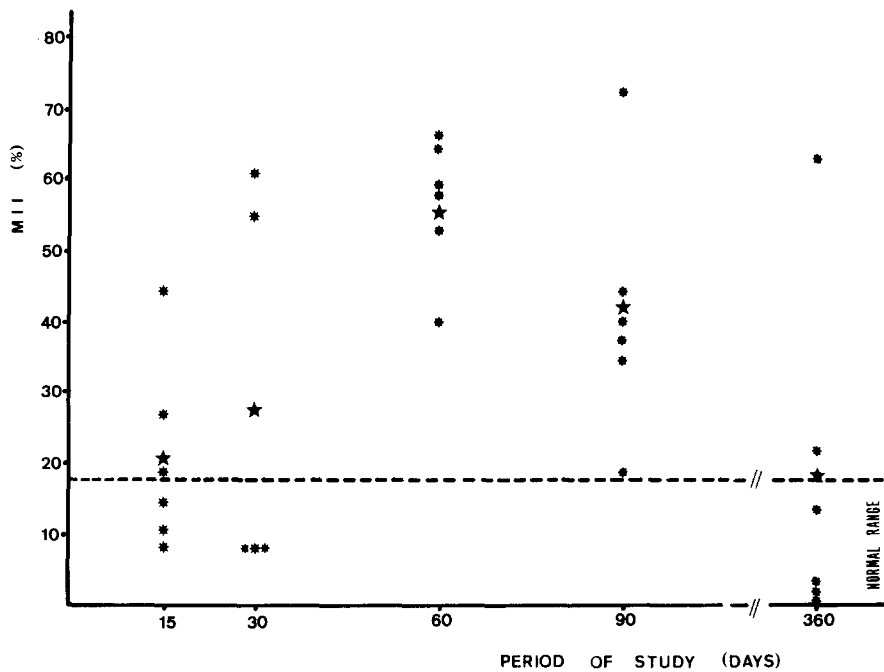

Fig. 5. MIF assay: response of cell-mediated immunity with PbAg (300 μg of protein) in infected mice expressed by the migration inhibition index (MII). ★ Indicates the means values.

only in 2 at Day 360 (33.3%). The strongest responses were observed at Day 60 and 90.

Immunodiffusion test. As shown in Table 4, specific antibodies were first detected at Day 15; the number of mice with detectable humoral response and the titers were maximal at Day 30 and 60. After one year of infection, the serology became negative.

Discussion

Experimental model

The present paper describes a murine model of pulmonary Pbmycosis, employing the direct intra-tracheal injection recently reported by Lawrence *et al.* (21).

The first step for the standardization of the model was the i.t. injection of a dye solution in normal animals; this experiment demonstrated that both lungs were stained by the dye. Even knowing

Table 4. Immunodiffusion test: number of mice with anti-*P. brasiliensis* antibodies distributed according to the titers.

Titers*	Experimental periods (days)					
	1 to 9	15	30	60	90	360
Negative	8	10	4	5	8	12
2	0	1	0	2	1	0
4	0	1	4	0	2	0
8	0	0	3	3	1	0
16	0	0	1	1	0	0
32	0	0	0	0	0	0
64	0	0	0	1	0	0
% of positive animals	0	16.7	66.7	58.3	33.3	0

* Expressed as the highest serum dilution reciprocal giving precipitation band.

that the distribution of a large particle such as a yeast cell of *P. brasiliensis* might probably be different from that of droplets of a soluble dye solution, the results of this experiment was taken as an indirect evidence that the pulmonary infection would be obtained. Sooner after the injection, a small amount of the dye leaked out from the trachea throught the needle orifice staining the paratracheal tissues; this was minimized by verticalizing the animals during the injection.

The second step was i.t. injection of 6×10^5 fungi per animal from a strain of Pb of known pathogenicity for the mouse (27). Three findings of the induced infection should be emphasized: 1) All animals developed pulmonary Pbmycosis up to Day 30. The high reproducibility of the infection was probably related to the fact that most of the injected fungi reached the alveoli. This is in contrast with the incidence of 34% (22) and 38.1% (31) of lung Pbmycosis lesions reported in mice infected by the intranasal route. 2) Part of inoculum flew back from the needle orifice which determined paratracheal specific lesion in 38.4% of the infected mice. This finding should be taken into consideration in experiments aiming at isolated pulmonary lesions of Pbmycosis. 3) In most of the mice some dye regurgitated to the oral and nasopharingeal cavities which most probable also occurred with the inoculum; even so, no specific lesions were found at those mucosae. This negative finding, also observed in mice infected by intranasal route (22), seems to indicate that unknown factors prevented the animals from the active intra-epithelial parasitism which has been described in human Pbmycosis and considered to be important in the oral route of infection (4).

Cell-mediated immunity

In the present infection, CMI responses were measured by both footpad test and MIF assay. Cell mediated immune responses were clearly detected in Pb-infected mice. Both tests gave positive and persistent responses since Day 15 up to Day 90. As in other models, histological reading of the footpad test showed higher sensitivity as compared with the swelling measurement (14). Footpad histological positive responses were only demonstrated in 20.5% of the infected mice as opposed to 43.5% of positivity in the MIF assay. This may be explained by the more complex mechanisms involved in the 'in vivo' expression of CMI (footpad test) than in its 'in vitro' counterpart (MIF assay) (7).

The time for the development of cellular immunity in fungi-infected animals is reported to be constant, between 10 to 15 days, as it was observed in the present model in which MIF-CMI responses were persistent up to the 360th day of infection. The persistence of the CMI responses has been reported to be variable (13, 19, 20, 24, 27, 32), depending on several factors, as the amount and via of administration of the inoculum, the virulence and antigenicity of the parasite and the host individual response (7).

Humoral immunity

Specific antibody response was detected since Day 15, peaked from Day 30 to 60, and was not observed at Day 360. The humoral response was similar to what is reported for guinea-pig (3) and mouse (27). Like in those experiments, the present infection showed a limited course with highest antibody titers coinciding with the most active phase of the disease. Even during this phase, the antibody production was small; this may be related with the limited extension of the infection, which was not seen beyond the pulmonary regional lymph nodes. Accordingly, increase in the antibody titers usually reflects severity of the mycotic infection, as it has been reported in human (9) and experimental (15) Pbmycosis, and in coccidioidomycosis, cryptococcosis and histoplasmosis (17).

Histopathology

Up to the 3rd day of infection the pulmonary inflammatory process was characterized by confluent foci of bronchopneumonia with great number of fungi. Linares & Friedman (22) described identical histological findings in mice inoculated with Pb by intranasal route; acute PMNs inflammation has been also observed in early experimental histoplasmosis, blastomycosis and coccidioidomycosis (33). On the other hand, it is noteworthy that some intraalveolar PMNs exudation was also seen in control mice, injected with sterile saline solution.

The histological pattern observed on the 15th day of infection was that of a desquamative macrophagic pneumonia, which has also been reported in patients with Pbmycosis (10). Teixeira *et al.* (34) described the same pattern in rats infected with Pb by intravenous route and sacrificed on Day 30. In the present model, the intensity of this intraalveolar inflammation seemed to be out of proportion with the number of fungi. Brito & Fava Netto (3) reported the same in pulmonary lesions of Pb-intravenous infected guinea-pigs and interpreted the lesions as due to hypersensitivity. As the same pattern of pneumonitis has been described in sensitized animals challenged by aereal route with different specific antigens (2, 29), it is presumable that the 15 day-old lesions may be related to a local Pb-cell-mediated immunologic reaction.

In the subsequent periods of sacrifice (Days 30, 60, 90), the number of specific lesions in the lungs decreased; the remaining foci were well circunscribed, characterized by epithelioid granulomata, crowned by lymphocytes and plasma cells and containing few fungi. The first appearance of granulomatous inflammation during the course of experimental Pbmycosis has been reported to be variable since Day 5 – intratesticular route in the hamster (15) and the guinea-pig (3) – to day 15 – intranasal route in the mouse (22). The kinetics of the inflammatory process since the initial non-specific phase up to the development of granulomatous inflammation is a complex mechanism, depending on the infectious agent, inoculum and host response (1). These variables make difficult a comparison between reported data on different experimental models of a particular infection.

In this work, strong CMI responses, as detected by MIF assay, and epithelioid granulomata occurred coincidently during the experiment; this finding, together with similar reports in several other infections (13, 18, 20, 32), suggests that cell-mediated immune response may have relevant role on the morphogenesis of paracoccidioidomycotic granulomata.

The present model of Pbmycosis was not a progressive infection; after one year most animals had no lung specific lesions. In addition, metastatic foci of the infection were observed only in regional lymph nodes in few mice, which contrasts with the higher incidence and extension of dissemination in other experimental mouse models of Pbmycosis (22, 31).

This may be explained by one or more of the following facts: natural resistance of mice against Pbmycosis (22, 27); low virulence of the Pb strain used; different route of infection. In addition, we believe that in our model the development of cellular immunity was effective in mediating a granulomatous inflammation which was able to confine and to heal the induced pulmonary Pbmycosis.

Acknowledgement

We are grateful to Mrs. Luiz G. Chamma and Wilson V. Fábio for their technical help during the course of this investigation.

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