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HISTOPATHOLOGICAL ALTERATIONS INDUCED BY NON-VIABLE CELLS AND BIOCHEMICAL FRACTIONS FROM *PARACOCCIDIOIDES BRASILIENSIS* IN MICE

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Non-viable cells and biochemical fractions from Paracoccidioides brasiliensis *were obtained for experimental inoculation in mice and posterior histopathological analysis. Dead total fungus, total fungus disrupted by sonorous waves, lipids of the fungus, supernatant of the lipid purification, integral and disrupted fungus free of lipids were obtained. The six preparations arised from masses of lyophilized yeasts of a recent isolate of* P. brasiliensis *(strain JT-1) and from a "Pool" equitably constituted by four strains maintained in laboratory for a long time (SN, 2, 18 and 192). Different doses of the 12 preparations were intraperitoneally inoculated and histopathological analysis were done 30 days later. This analysis showed that all the inoculated preparations gave origin to inflammatory foci, except the one designated "supernatant of lipid purification". The alterations were detected exclusively in the liver of the animals and occurred from the smallest dose tested (1 mg), with exception of the lipids of the fungus, where the foci appeared only from a 3 mg dose onwards. No difference in the capacity of inducing histopathological alterations was found between the preparations obtained from the recent isolate (JT-1) and from the older ones ("Pool"). On the other hand, an increase of the number of inflammatory foci in function of the inoculated dose was observed.*

Key words: *Paracoccidioides brasiliensis* – biochemical fractions of *Paracoccidioides brasiliensis* – histopathological alterations

Paracoccidioidomycosis is a human infection caused by the dimorphic fungus *Paracoccidioides brasiliensis*. This disease is endemic in Latin America where it presents the deep mycosis of major occurrence. Brazil is the endemic center with the highest number of cases so far, followed by Colombia, Venezuela and Argentina (Hamdan & Rocha, 1987; Moreno, 1990; Lacaz, 1991).

Although a high number of researches and publications has been made on this mycosis and its etiological agent, several aspects related to the pathogenesis of this disease still have to be elucidated. Much is still unknown, from the habitat of the fungus up to the determining steps of the different manifestations and evolution forms of the mycosis (Hamdan & Rocha, 1987; San-Blás & Castaneda, 1990).

The spectrum of the disease can range from an asymptomatic benign pole up to a malignant pole, presented by severe, progressive and/or fatal forms. Various intermediate manifestation forms could be detected between the two poles (Franco, 1987; Franco et al., 1988; Conti-Diaz & Rodriguez, 1990; San-Blás, 1990). Factors related to the host, the environment, and the microrganism are being blamed not only for the different presentations of the disease (Franco, 1987) but also for the diversity of the frequency of the several clinical forms observed in different regions of a same country or in different countries (Montenegro et al., 1988).

As to the microrganism, there is a consensus about the existence of different strains of the only species described. These differences have already been demonstrated in animals, in terms of degrees of virulence (Kashino et al., 1985, 1987; Zacharias et al., 1986; Singer-

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Vermes et al., 1989) and have been associated with peculiar biochemical characteristics of *P. brasiliensis* strains (San-Blás & San-Blás, 1977; Zacharias et al., 1986; San-Blás & Castañeda, 1990). On the other hand, biochemical fractions of the fungus have been related to the genesis of the inflammatory response in paracoccidioidomycosis. It has been demonstrated that cell wall polysaccharides and lipids, on their own, can induce granulomas when inoculated experimentally (Silva, 1985; Silva & Fazioli, 1985a; Alves et al., 1987; Franco, 1987).

With the aim to contribute to the knowledge of the pathogenesis of paracoccidioidomycosis, the present work studies the effect of inoculation in mice, of non-viable cells and different biochemical fractions of the fungus and compares the capacity to induce histopathological alterations of preparations from different *P. brasiliensis* strains.

MATERIALS AND METHODS

Microrganisms – The *P. brasiliensis* strains used in this study performed four samples (SN, 2, 18 and 192) obtained from the collection of the Faculty of Medicine of the University of São Paulo and maintained in laboratory for a long time, as well as a recent isolate of this fungus (JT-1), identified in the Laboratory of Mycology, Federal University of Minas Gerais, in January, 1990.

Cultivation – The five strains were cultivated in yeast phase (36 °C) in solid synthetic medium of McVeigh & Morton (Restrepo & Jimenez, 1980). Subcultures done in four or five day intervals provided cells in exponential growth phase (Manocha, 1980; Manocha et al., 1980). These cells were carefully collected using a loop, transferred to the centrifuge, washed three times with a sterile saline solution and, then, frozen and lyophilized. The strains SN, 2, 18 and 192 were equitably combined in order to obtain a pool.

Viability test – Aliquots of the lyophilized masses were studied as to cell viability by fluorescence test as described by Calich et al. (1978).

Partial biochemical characterization of the strains – Lyophilized cell masses of the strain JT-1 and of the pool, obtained as described in *Cultivation,* were analyzed as to the total lipid and carbohydrate contents. Lipids were extrated and purified by the method of Letters (1968) modified by Hunter & Rose (1972). The total lipid content was determined by dry weight, after evaporation in vaccum (Hamdan & Resende, 1988). Carbohydrates were measured after sonication of the lyophilized cell masses in MSE equipment. Ninety cicles of thirty seconds each were done, at a medium intensity and amplitude 4. Under these conditions, 90% of cell rupture (Restrepo et al., 1984), observed by microscopy, was obtained. Total carbohydrate content was determined by the spectrophotometric anthrone technique (Scott & Melvin, 1953).

The results of all measurements were expressed as mg of substratum/100 mg of lyophilized cells (mg%) and represent the mean and standard deviation of at least three experiments carried out for both studied samples (JT-1 and "Pool"). The "t" student test was used to determine statistical differences in the total lipid and carbohydrate contents of the two samples.

Preparations of the fungus – Six preparations of the fungus were obtained for experimental inoculation from the lyophilized masses of the recent isolate (JT-1) and six preparations from a pool of four previous isolates (SN, 2, 18 and 192). The six preparations comprised: (1) Total integral fungus. (2) Total fungus disrupted by sonorous waves where the cell masses were weighed, resuspended in phosphate buffered saline (PBS) and submitted to sonication in MSE equipment, as described above. (3) *P. brasiliensis* lipids. Purified lipid extracts, from the samples JT-1 and "Pool" were obtained as previously described and lyophilized. (4) Supernatant of the lipid purification. The purification phase of the lipids extracted from *P. brasiliensis* gave origin to a biochemical fraction corresponding to the non-lipid material "dragged" in the extraction process. This material was lyophilized and designated "supernatant of the lipid purification". (5) Integral fungus free of lipids. The remainder cells of the lipid extraction were lyophilized and formed the fraction designated "integral fungus free of lipids". At microscopy, aliquots of this preparation showed the integrity of the yeasts, although part of them (approx. 30%) had clearly lost their cytoplasmic content. (6) Fungus free of lipids disrupted by sonorous waves. The fraction previously described was submitted to cell rupture as mentioned in item (2).

Preparation of the inocula – The 12 preparations obtained (six for the sample JT-1 and six for the "Pool") were, after lyophilization, stocked at –20 °C. The inocula of each preparation were made by obtaining a suspension in sterile PBS containing, for example, 5 mg of the substratum per ml of suspension. At the time of inoculation, the suspensions were further diluted in sterile PBS to obtain the wanted doses.

Experimental model – The experimental model used in this study comprised white, non isogenic, male mice (*Mus musculus*) with age from seven to eight weeks. Twelve groups were formed, corresponding to the twelve preparations studied. Each group contained 12 animals, nine were treated and three remained control.

Inoculated doses – Each dose of all preparations were inoculated in triplicate (three mice). The doses chosen were 1, 3 and 5 mg for all preparations, except the one designated "supernatant of lipid purification". The latter, being a fraction obtained in very small quantities, was inoculated in smaller doses, i.e., 0.5, 0.75 and 1 mg.

Inoculation procedure – The inoculations were done intraperitoneally and the inocula were composed of 1 ml of suspension of preparations in the treated groups and 1 ml of diluent (PBS) in the control group.

Follow-up and sacrifice – Each group of inoculated mice was kept under observation for 30 days. At the end of this period the animals were sacrificed under deep anesthesia, through ether inhalation, followed by bleeding by cutting the axillar artery.

Preliminary experiments – Prior to the group formation, inoculation, follow-up and sacrifice, preliminary experiments were made to elect a suitable period of follow-up and sacrifice of the animals. A group of 12 mice received, each three, the highest tested dose (5 mg) of each one of the following preparations obtained from the pool of *P. brasiliensis* strains: total fungus disrupted by sonorous waves, lipids, and fungus free of lipids disrupted by sonorous waves. The three remainder animals represented the control of this preliminary experiment. Each one of the three mice inoculated with each preparation was sacrificed, respectively, after 7, 15 and 30 days post-inoculation. At the same intervals, each one of the controls was sacrificed.

Histopathological study – All mice were necropsied and liver, spleen, kidneys, intestine, peritoneal membrane, diaphragm and lungs were removed. The samples were fixed in 10% formalin, embedded in paraffin, slices were cute in 5 μm thick and stained by hematoxylin-eosin for microscopic analysis. Histopathological examination was aimed at detecting specific (granuloma) and non-specific (congestion and hemorrhage) alterations. The alterations observed were semiquantified. In case of presence, they were classified in low, medium and high intensity for the phenomena congestion and hemorrhage. In relation to the inflammatory foci, the mean was calculated according to the number counted in three sections, being classified in rare (+), medium (++) or numerous (+++). The signs represent 1 to 3, 3 to 6 and more than 6 foci, respectively. In all the cases, the changes were recorded when they occurred in at least two of the three mice inoculated with each dose.

RESULTS

The viability tests done in the preparation "total integral fungus" did not show any viable cells. Therefore, this study does not refer at any moment to the infection caused by *P. brasiliensis*, but only to the capacity of different preparations to induce tissular inflammatory responses.

Total lipid and carbohydrate contents of the samples JT-1 and "Pool" are shown in Table I. Comparison of the means of the measurements by the "t" test showed a higher amount of lipids and carbohydrates in the strain JT-1 ($p < 0.01$).

TABLE I

Total lipid and carbohydrate contents (mg%) of a pool of four strains (SN, 2, 18 and 192) and of the sample JT-1 of *Paracoccidioides brasiliensis* in yeast phase

Strains	Lipids		Carbohydrates	
	$\bar{X}$	s	$\bar{X}$	s
"Pool"	7.0	0.00	26.5	0.71
JT-1	9.7[1]	0.53	52.4[2]	1.02

$\bar{X}$: average of at least three experiments.
s: standard deviation.
1: higher than pool; $t = 7.3$, $p < 0.01$.
2: higher than pool; $t = -29.43$, $p < 0.01$.

Preliminary experiments revealed that significant histopathological alterations have not occurred up to the 15th day post-inoculation. Therefore, a period of 30 days was chosen for the follow-up and sacrifice of the 144 animals which formed the experimental group in this study.

No macroscopic alterations of the viscera were detected in any of the treatments. The results described hereafter represent microscopical changes only.

Focal inflammatory processes were composed of mononuclear cells, the latter almost exclusively represented by macrophages. They were not defined as granulomas because of the non-characteristic arrangement of these cells as well as their small size. These observations are referred to as "inflammatory foci of mononuclear cells" and were detected exclusively in the liver of the animals, distributed through the parenchyma or, frequently, around the vessels. Some of these structures are shown in Figs 1 and 2. All the inoculated preparations gave origin to inflammatory foci, except the one designated "supernatant of lipid purification". This alteration was observed from the smallest dose tested (1 mg), with exception of the lipids of JT-1 and "Pool", where the foci appeared only from a 3 mg dose onwards. Results are shown in Tables II, III and IV. The other preparations are not graphically represented since they follow the same pattern as shown in Table II. Besides, an increase of the number of foci in function of the inoculated dose was observed. None of the 30 control mice presented this change.

Congestion and hemorrhage were frequently detected in different organs of the mice inoculated with the 12 preparations (Tables II, III and IV). Congestion occurred also in one or another viscera of the control group and hemorrhage was not significantly observed in these animals.

TABLE II

Semiquantified histopathological alterations observed after inoculation of Preparation 1 : Total integral fungus ("Pool")

Dosis (mg)	Inflammatory foci	Congestion Intensity/ Viscera	Hemorrhage Intensity/ Viscera
1.0	+	+ Lung Kidney	0 –
3.0	++	+ Liver Lung	+ Lung
5.0	++	+ Liver Lung Kidney Spleen	+ Lung

0: no alterations.
+: 1 to 3 inflammatory foci or small areas of congestion and/or hemorrhage.
++: 3 to 6 inflammatory foci or medium areas of congestion and/or hemorrhage.
+++: more than 6 inflammatory foci or big areas of congestion and/or hemorrhage.

TABLE III

Semiquantified histopathological alterations observed after inoculation of Fraction 6 : Lipids (Strain JT-1)

Dosis (mg)	Inflammatory foci	Congestion Intensity/ Viscera	Hemorrhage Intensity/ Viscera
1.0	0	+ Kidney	+ Lung
3.0	+	+ Lung	0 –
5.0	++	+ Lung Kidney	+ Lung -

0: no alterations.
+: 1 to 3 inflammatory foci or small areas of congestion and/or hemorrhage.
++: 3 to 6 inflammatory foci or medium areas of congestion and/or hemorrhage.
+++: more than 6 inflammatory foci or big areas of congestion and/or hemorrhage.

TABLE IV

Semiquantified histopathological alterations observed after inoculation of Fraction 7 : Supernatant of lipid purification ("Pool")

Doses (mg)	Inflammatory foci	Congestion Intensity/ Viscera	Hemorrhage Intensity/ Viscera
0.50	0	+ Liver Lung	0 –
0.75	0	+ Lung Kidney	0 –
1.00	0	+ Lung Kidney	0 –

0: no alterations.
+: 1 to 3 inflammatory foci or small areas of congestion and/or hemorrhage.
++: 3 to 6 inflammatory foci or medium areas of congestion and/or hemorrhage.
+++: more than 6 inflammatory foci or big areas of congestion and/or hemorrhage.

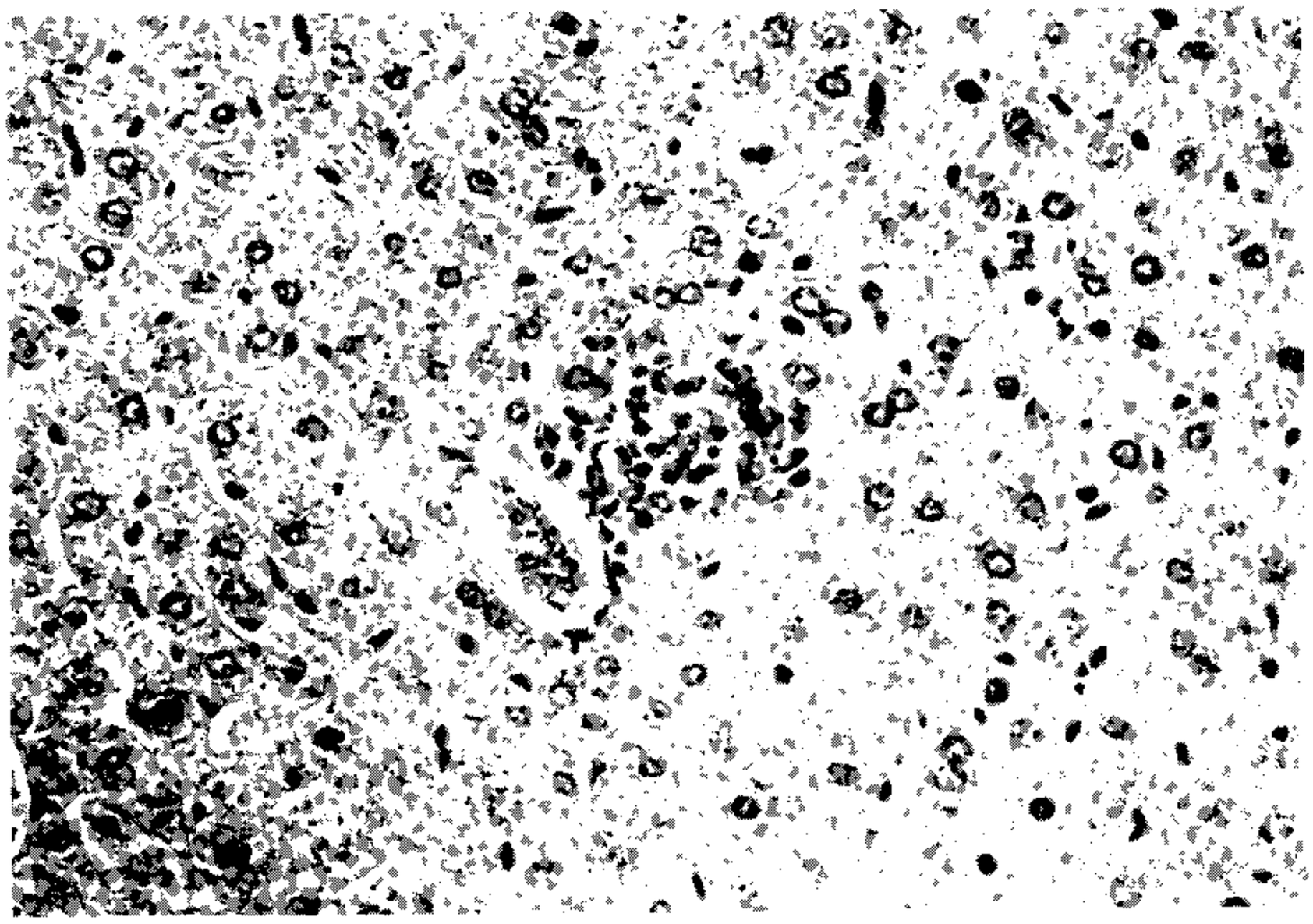

Fig. 1: liver. Perivascular inflammatory focus of mononuclear cells (HE X 200).

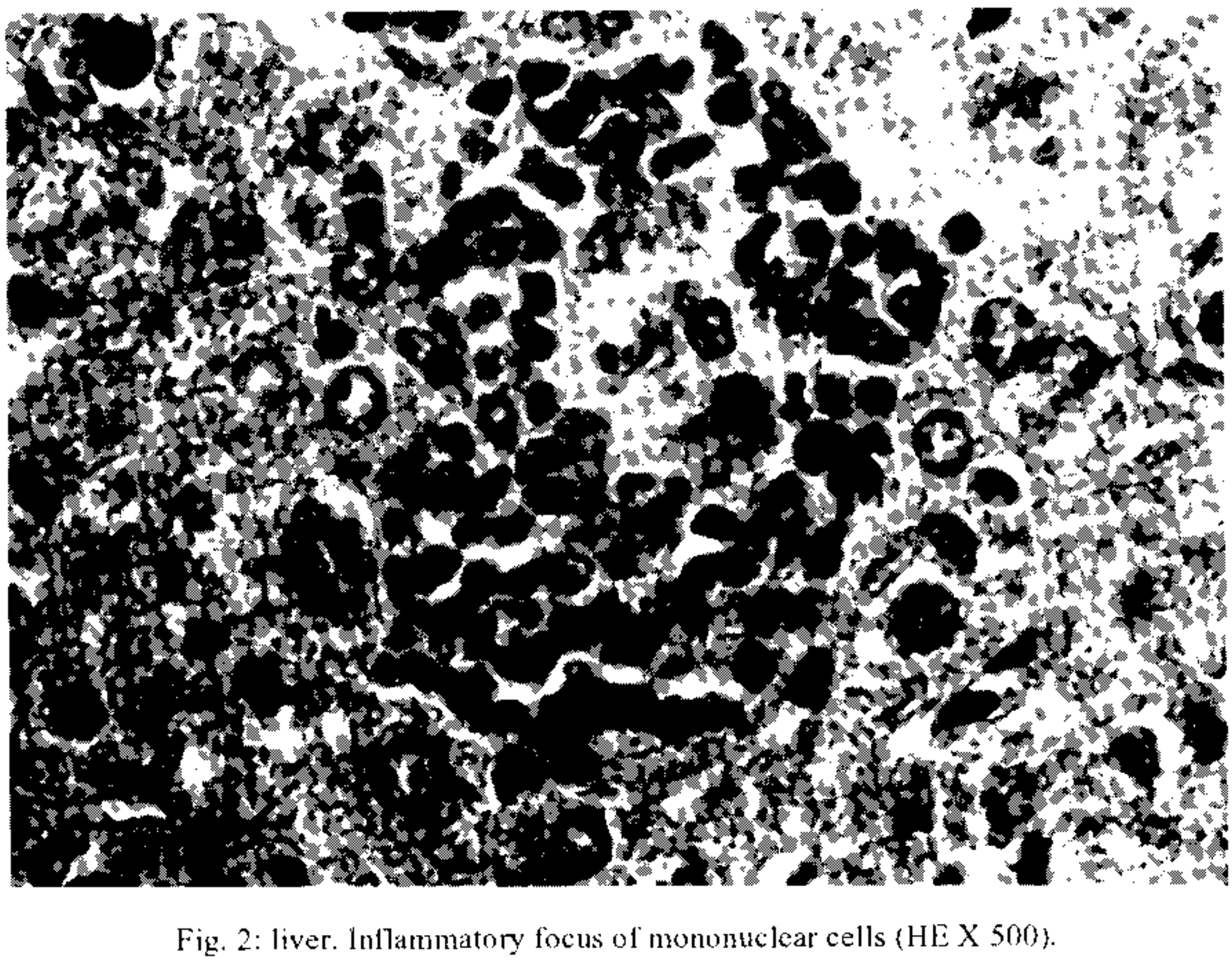

Fig. 2: liver. Inflammatory focus of mononuclear cells (HE X 500).

DISCUSSION

The granuloma represents the histological pattern typical of infection caused by *P. brasiliensis* (Franco, 1987; Franco et al., 1988; Moreno, 1990). This structure has frequently been induced in experimental models inoculated with this fungus. However, there are conflicting reports as to its observation due to, among others factors, the route of inoculation (Moscardi & Franco, 1980; Singer-Vermes et al., 1989). On the other hand, it has been shown that biochemical fractions of *P. brasiliensis* and of fungi agents of cromoblastomycosis, are inducers of granuloma when inoculated intravenously or intraperitoneally in mice or rats. These fractions correspond to the lipid of the microrganisms, to cell wall polysaccharides,

to fungus free of lipids disrupted by sonorous waves and to dead *P. brasiliensis* (Silva, 1985; Silva & Ekzlerian, 1985; Silva & Fazioli, 1985a, b; Alves et al., 1987).

In this study, the hepatic inflammatory foci found in association with the inoculation of the preparations described and of others not referred to yet in the literature, were not considered as granulomas due to the non-characteristic arrangement of the cells. However, some authors believe that the presence of macrophages and lymphocytes, observed in the foci found, is sufficient to designate them granuloma structures.

As to the capacity of provoking a tissue reaction of the host, the results found agree with what was previously published in the literature for total non-viable fungus, fungus free of lipids an lipids purified of *P. brasiliensis.* It was also observed that cell rupture, liberating the cytoplasmic content, did not increase the capacity of inducing inflammatory foci in relation to the integral preparations. This observation, as well as the one referring to fungus free of lipids, integral or disrupted, which also induced inflammation, confirm reports of authors mentioned previously, that cell wall polysaccharides are involved in the genesis of the tissue response of the host. Likewise, the results referring to the purified lipids agree with these reports.

The fraction designated "supernatant of lipid purification" was the only one which has not induced inflammatory foci, probably in function of the small dosis inoculated and, in correspondence, the low concentration of carbohydrates which, comprehensibly, is minute in this fraction, as well as the total absence of lipids (Hamdan, 1991).

No difference in the capacity of inducing histopathological alterations was found between the preparations from the recent isolated sample (JT-1) and from the older ones ("Pool"). The partial biochemical characterization of the pool, equitably composed by the strains SN, 2, 18 and 192, showed that the sample JT-1 has a much higher carbohydrate content than "Pool". Since the polysaccharides are associated to the genesis of the granuloma as mentioned, it was expected that this strain and its fractions would be more efficient as to induce inflammatory foci than the "Pool". However, this was not observed. The findings suggest that the total content of polysaccharides might be less important, in relation to the capacity of causing tissular response, than the presence of specific carbohydrates among these polysaccharides.

In relation to the non-specific alterations, it was not observed any association with the 12 inoculated fractions and congestion, since this phenomena was also found in the animals of the control group. Furthermore, an increase of the inoculated dosis has not correlated with the extent of this alteration. On the other hand, hemorrhage was not significantly observed in the control group, nor in the mice treated with the fraction "supernatant of lipid purification", which has not induced any inflammatory foci either. This fact suggested association of this phenomena with the inoculated preparations that were able to induce inflammatory foci in the liver of the mice. However, this alteration was not always present and its extent has not showed correspondence with the increase of the inoculated dosis. In addition, hemorrhage has not occurred in specific organs, as observed with the inflammatory foci, that was found only in the liver of the treated mice. These inconstant findings and also, the infinity of causes that may be related to congestion and hemorrhage, have not allowed association of these phenomena with the inoculation of different preparations of the fungus.

The data presented, as well as those previously reported in the literature, show the relevance and necessity of further studies on biochemical fractions of *Paracoccidioides brasiliensis* in an attempt to throw light on the pathogenesis of paracoccidioidomycosis.

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