ORIGINAL INVESTIGATION

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Influence of the genetic background on the pattern of lesions developed by resistant and susceptible mice infected with *Paracoccidioides brasiliensis*

Abstract To compare the sequential evolution of lesions developed by resistant (A/Sn) and susceptible (B10.A) mice to *Paracoccidioides brasiliensis* infection we inoculated a virulent isolate of the fungus and collected the pancreas/peripancreatic omentum monthly (from 1 to 6 months) post infection. After fixation, tissue sections were stained by conventional methods for light microscopy to investigate the cellular composition, the extracellular matrix (ECM) patterns and the morphology of the yeasts in the lesions. In both strains, the fungal lesions were localized mostly in the omentum; a few lesions in the pancreatic parenchyma were observed, mostly in B10.A mice. In both strains, macrophages and plasmocytes were the predominant cells in all lesions, followed by neutrophils (PMN) and macrophages transformed into giant and epithelioid cells. Remarkable differences were observed between resistant and susceptible mice, specially related to the ECM structure of the granulomatous lesions. In A/Sn mice, from the 1st month on, the coexistence of two types of lesions was observed: one type showed a well-defined encapsulated nodule, constituted mainly of type I collagen. Neutrophils were abundant in areas of massive fungal destruction and few viable yeasts were observed. The other type showed residual characteristics, with sparse collagen deposits and presence of xantomatous-like macrophages, containing degenerated fungi. Such residual lesions predominated after the 2nd month and were the only type observed from the 4th month on, indicating the control of the infection. In B10.A mice, on the contrary, only one type of lesion was observed, showing less tendency to encapsulation and the formation of multiple small granulomatous foci, individualized by reticular type III collagen fibers. There were many plasmocytes in the periphery and large numbers of budding yeasts, with no evidence of fungal destruction. In the course of the infection the lesions progressively increased in number and size. Altogether, the comparative histopathological analysis demonstrates the influence of the genetic pattern of the host on the lesions developed by resistant and susceptible mice to *P. brasiliensis* infection.

Key words Paracoccidioidomycosis · Histopathology · Granulomatous lesions · Inbred mouse strains

Introduction

Paracoccidioides brasiliensis is a fungus restricted to Latin America that causes one of the most prevalent human deep mycosis in this region [29]. Although the lungs are the organs primarily involved in paracoccidioidomycosis (PCM), dissemination to other tissues is commonly found, depending on aspects inherent to both the host (sex, age, nutritional state) and the infecting agent (virulence, size of the inoculum) [13]. PCM is a granulomatous disease and may present several clinical forms. The chronic form, the adult type of PCM, is the more frequent manifestation of the disease, in which several degrees of severity are observed [13]. Patients manifest depressed cellular immune responses that represent the main defense mechanism in PCM and high activation of the humoral compartment, to a greater or lesser extent, in accordance with the severity of the disease [2, 14, 27].

In an isogenic murine model of PCM, Calich et al. [5] demonstrated differences in susceptibility to *P. brasiliensis* among inbred strains, related to their genetic pattern. A/Sn mice were resistant and B10.A mice sus-

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ceptible to intraperitoneal (i.p.) infection with the virulent isolate Pb18 of *P. brasiliensis*. The mean survival times were 283 and 124 days, respectively. However, a few susceptible mice survived until 200 days. Resistance to the fungus seemed to be controlled by a dominant autosomal gene [6], and the F1 hybrids of the breeding of B10.A and A/Sn mice were resistant to *P. brasiliensis*. After Pb18 i.p. infection, the analysis of several parameters indicated that resistant A/Sn and susceptible B10.A mice developed disease with characteristics similar to those observed, respectively, in the localized and disseminated forms of chronic human PCM [7]. In resistant mice the infection was characterized by a high activation status of peritoneal macrophages and polymorphonuclear neutrophils (PMN), evidenced by the increase of hydrogen peroxide (H_2O_2) production, and maintenance of superoxide anion (O_2^-) production, low titers of total specific anti-*P. brasiliensis* antibodies, with preferential production of IgG2a and IgG3 isotypes [8], absence of circulating *P. brasiliensis* antigens [15], and evident specific delayed-type hypersensitivity (DTH) [12] were also detected. On the other hand, in susceptible mice the macrophages and PMN produced low levels of H_2O_2 and O_2^-, large quantities of antibodies (predominantly the IgG2b, IgG1 and IgA isotypes) [8], increasing levels of antigenemia [15], and depression of specific DTH reactions to the fungus [12].

In this model, the involvement of the pancreas and the peripancreatic omentum, liver, spleen and lungs was quite frequent in both susceptible and resistant mice, as observed in anatomicopathogical studies [5]. These features were corroborated by the number of recovered viable *P. brasiliensis*: in B10.A the number of colony-forming units (CFU) from these organs was always higher than those observed in A/Sn mice from weeks 2 to 16 after Pb18 i.p. infection, when the lesions were present only in the susceptible B10.A mice [31]. Concerning the pancreas involvement, the infection of B10.A mice with Pb18 evoked a significant increase in their plasmatic amylase levels, a phenomenon not observed in the A/Sn mice or in the F1(B10.A × A/Sn) hybrids, thus allowing the establishment of a positive correlation between the increase of amylase and the susceptibility of these animals to the fungus [34]. Differences in the pattern of histopathological lesions were also observed in these animals. In general, B10.A mice showed abundant and poorly organized granulomatous lesions rich in yeasts and plasmocytes, scattered in the tissue. In contrast, in A/Sn mice only few granulomas were detected, presenting scarce fungal cells and plasmocytes [8].

In PCM, as in other infectious diseases (schistosomiasis, leprosy, tuberculosis and listeriosis), the formation of granulomas constitutes a delayed hypersensitivity reaction to antigens from the infecting agent. This reaction is greatly affected by cytokines influencing both the constituent cells and the extracellular matrix (ECM) fibers present in the lesions. Several cytokines [interleukin (IL)-1, IL-6, granulocyte-macrophage colony-stimulating factor] participate in the early inflammatory reaction, promoting extravasation of leukocytes and expression of adhesion molecules by endothelial cells [22]. Some cytokines, such as transforming growth factor (TGF)-β, are strongly associated with the depressed cellular immune responses observed in granulomatous infectious diseases; its enhanced production by pulmonary macrophages and Langhans' giant cells in patients with active tuberculosis, counterbalance the macrophage stimulation by tumor necrosis factor-α and interferon (IFN)-γ [32]. In the murine model of pulmonary PCM, it was observed that the depletion of IFN-γ gives rise to an increase in the severity of the granulomatous lesions, in both resistant and susceptible mice, thus indicating the role of this cytokine in the development of this pathology [9]. The effect of ECM proteins on inflammatory cells was studied using human macrophages adhered to collagen 1 gel matrices, which showed an increased fungistatic activity against *Histoplasma capsulatum* yeasts [28]. Furthermore, the activity of proteolytic enzymes of the infecting agents may modulate the deposition of ECM fibers in the granuloma development [30].

In the present work the evolution of the granulomas in a relatively dense organ (pancreas) and in the loose connective tissue (peripancreatic omentum) was compared during the course of i.p. infection of susceptible and resistant mice with a virulent strain of *P. brasiliensis*. The study was performed analyzing the cellular composition and fibrotic architecture of the lesions, as well as the ''in situ'' effect of the fungus, taking into account the influence of the genetic pattern of the host on the establishment and development of the tissular reactions to *P. brasiliensis*.

Material and methods

Animals

Groups of 8- to 10-week-old males mice were used. For the experiments using resistant A/Sn mice, groups of three to five animals were employed for each data point. For the experiments with susceptible B10.A mice, larger groups were formed to ensure that sufficient number of mice survived until the 6th month of infection. The mice were bred at the Departamento de Imunologia, Universidade de São Paulo animal facilities and fed with laboratory chow (Nuvilab CR-1, Nuvital) and acidified water ad libitum.

Fungus

The yeast forms of *P. brasiliensis* virulent isolate Pb18, cultivated in semi-solid Fava Netto's [11] medium at 35 °C were employed at the 7th day in culture, which corresponds to the exponential phase of growth [19]. To prevent loss of virulence, every year a new sample of Pb18 is obtained after in vivo passage and stored in its micellar form in potato dextrose agar; Pb18 yeasts are maintained in sequential in vitro subcultivation for no longer than 3 months [20]. The yeast cells were washed in isotonic sterile saline and the fungal suspensions were employed at a concentration of 10×10^6 yeast cells/ml, adjusted using an hemocytometer. The viability of the fungal cells was evaluated using the vital dye Janus Green B [1], and was always higher than 80%.

Infection

The mice were inoculated i.p. using 5×10^6 fungal cells suspended in 0.5 ml isotonic sterile saline solution. This dose was employed since previous results showed that it was an adequate inoculum to differentiate susceptible from resistant mice. Control groups of mice were inoculated with 0.5 ml of isotonic sterile saline solution.

Collection of tissue samples

At 1–6 months after Pb18 infection mice were anesthetized with ethyl ether vapor, bled to allow better histological preparations and killed. The pancreas and lesser omenta (pancreaticosplenic and splenogastric regions) were removed and spread out in a piece of paper to preserve them extended. The organs were immediately fixed in Millonig formalin pH 7.2 [10], and maintained at 4 °C until processed.

Histopathological analysis

For light microscopy, the fixed tissues were embedded in paraffin and 5-μm sections were stained using the following methods: hematoxylin and eosin (H&E) for a general overview; Grocott's methenamine-silver nitrate technique [17] for fungi identification and viability evaluation; Lennert's Giemsa [21], for cellular infiltrate characterization and Masson's trichrome [23], Gomori's silver reticulin [16] and picrosirius, with or without light polarization [18] for identification of collagen fibers.

Evaluation of the severity of lesions

The pathological alterations observed in each mouse were semiquantitatively expressed according to the lesions' severity: 0, absence of lesions; 0.5, very discrete; 1, discrete; 1.5, discrete to moderate; 2, moderate; 2.5, moderate to intense; and 3, intense. This criterion was based on the number and size of granulomatous lesions, number of fungi with preserved morphology and the intensity of the inflammatory reaction. The number and size of the lesions were quantified employing a standardized reticulated objective (micrometer scale), allowing measurement of the area of each square. As the lesions showed irregular boundaries, their area was calculated summing the number of squares entirely filled by lesions with the half-number of squares partially occupied (approximate integral calculus).

Results

Preferential site of lesions

The lesions caused by Pb18 were preferentially found in the peripancreatic region, thus confirming previous observations that this loose connective tissue (epiploon) represents the target tissue after i.p. *P. brasiliensis* infections for both susceptible and resistant mice. The distribution and intensity of lesions found in the pancreas and peripancreatic omentum of A/Sn and B10.A mice is shown in Fig. 1. Susceptible mice showed a more intense involvement of both epiploon and pancreas than the resistant ones. In the pancreas, from 2 months on, the majority of the susceptible animals had intensities scored 2 or higher, whereas almost all the resistant ones had lesions varying from 1 to 1.5. This was even more marked for the epiploon, where from

Fig. 1 Individual data of fungal lesions in pancreas and epiploon of A/Sn (○) and B10.A (●) mice i.p. infected with 5×10^6 yeasts of *Paracoccidioides brasiliensis* isolate Pb18. The severity of lesions was semiquantitatively expressed as: *0* absence of lesions, *0.5* very discrete lesions, *1* discrete, *1.5* discrete to moderate, *2* moderate, *2.5* moderate to intense, and *3* intense. This criterion was based on the number and size of granulomatous lesions, number of fungi and the intensity of the inflammatory reaction
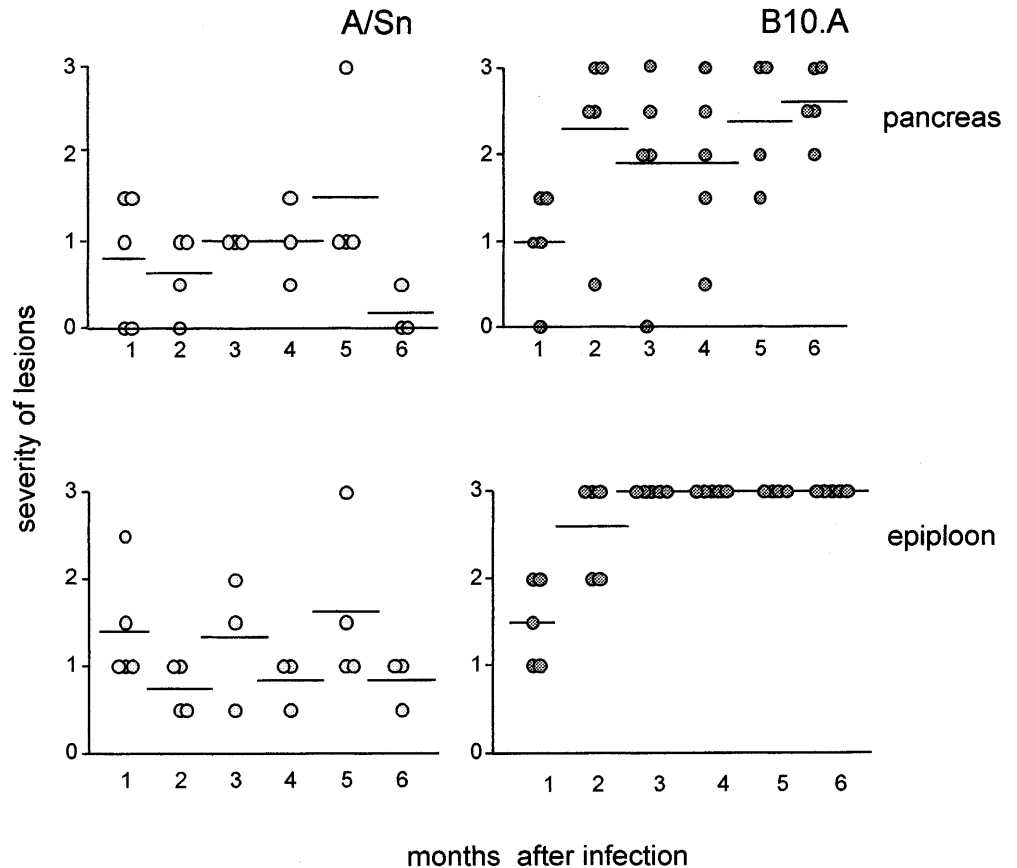
3 months on all susceptible mice scored 3, whereas only one mouse of the resistant group did so. The severity of the lesions found in the epiploon was higher than that found in the pancreas for both mouse strains. Recent, unpublished data of CFU per gram of pancreas and epiploon show a marked difference in the numbers of viable *P. brasiliensis* recovered from these organs. Already at 7 and 15 days after infection, a growth of 1406 and 1415 CFU/g tissue, respectively, was found in B10.A mice, in contrast to 0 and 71 CFU/g recovered from A/Sn mice. This difference in the number of viable yeast cells recovered from the two mouse strains was also observed in the later phases of the infection [31].

Omentum involvement

Histologically, the peripancreatic omentum presented fungal lesions in all the mice analyzed. In this region different patterns of granulomas were detected in resistant and susceptible hosts.

In resistant A/Sn mice two types of lesions could be distinguished. One type was an encapsulated, active lesion with deposition of ECM proteins, forming a dense fibrotic capsule, as shown in Fig. 2A, mainly composed by type I collagen fibers identified by picrosirius stain with polarized light (red/yellow fibers, Fig. 2B). In this type of lesion, foci of PMN were quite frequent, surrounding *P. brasiliensis* yeasts (Fig. 2C). We could also identify some *P. brasiliensis* yeasts with preserved morphology localized in the central area of the granulomas (Fig. 2D, left), free fungal debris in areas of neutrophil concentration (Fig. 2D, central area) and intracellular fungal debris in the external cellular layer of the lesion (Fig. 2D, right), in sections stained by Grocott's method. These types of granulomas were especially detected at 1 month post infection. The other type of lesion found in resistant mice was a non-capsulated, loose nodule with residual characteristics. These lesions were permeated by sparse collagen fibers, stained red by the picrosirius method (Fig. 2E); the same residual lesion is shown in Fig. 2F using polarized light, to give a better identification of the small amount of collagen fibers (stained in green/yellow), concentrated in the central area of the lesions. This type of lesion was mainly composed of xantomatous-like macrophages, denominated "pseudoxantomatous" macrophages (Fig. 2G). These cells containing degenerated fungi were evidenced by Grocott's stain (Fig. 2H). This type of lesion was already present at 1 month post-infection, simultaneously with the active lesions, became predominant from 2 months on, and was the unique kind of lesion present from 4 month onwards.

In susceptible B10.A mice only one type of lesion was observed in the course of *P. brasiliensis* infection, showing less tendency to define a capsule, and with little or absent type I collagen deposition, as showed in the Fig. 3A using picrosirius staining. These lesions could be defined as a nodule composed of multiple granuloma-tous foci, each surrounded by a thin layer of reticular collagen fibers, specially of type III, stained yellow/green by polarized light on picrosirius staining (Fig. 3B). The multiple granulomatous foci are shown in Fig. 3C, each of which was composed of macrophages and fungi. In the periphery of the lesion, large numbers of plasmocytes were always observed (Fig. 3D). In these lesions Langhans' giant cells, with many nuclei, were quite common, especially in the chronic phases after 4 months post-infection (Fig. 3E). Yeasts of *P. brasiliensis* with preserved morphology were found in large numbers, with abundant budding and insignificant levels of fungal destruction (Fig. 3F). The architecture of the multifocal granulomas was not altered during the course of the infection; however, with the chronicity of the disease, the granulomas became more expanded and reflected severe disease. In fact, in this experimental model after 5 months of infection the majority of the infected B10.A mice had died. Indeed, most deaths for this mouse strain occurred between 50 and 180 days, a much shorter time when compared to the resistant A/Sn mice, which died between 90 and 600 days post infection [8].

Pancreatic involvement

The histopathological analysis of the pancreas allowed us to conclude that from 2 months post infection an important impairment of the pancreatic parenchyma occurred in susceptible B10.A mice. In most mice of this strain, the lesions varied from those of moderate to those of severe intensity, whereas in the resistant A/Sn mice almost all lesions were discrete (only one A/Sn mouse had a severe pancreatic lesion, 5 months post-infection). Even in B10.A mice, the pancreatic tissue was mostly preserved, with almost all lesions being localized in the external lobules, which were in contact with the pseudocapsule of the pancreas. Figure 3G shows a discrete inflammatory infiltrate, in addition to a small granuloma, involving a few pancreatic acinar cells in A/Sn mice 3 months after Pb18 infection. In B10.A mice, the inflammatory reaction to the presence of *P. brasiliensis* was more intense, with destruction of several pancreatic lobules, preserving some atrophied ducts and cells as shown in Fig. 3H.

Fig. 2 A Active encapsulated lesion in peripancreatic omentum, with collagen fibers encircling inflammatory and fungal cells; picrosirius stain. **B** The same section, showing the collagen capsule (*red/yellow fibers*) of the lesion, under polarized light microscopy. **C** Foci of neutrophils surrounding Pb18 yeasts; H&E. **D** Pb18 in a granulomatous lesion: some viable yeasts (*left*), fungal debris (*center*), degenerated fungi within macrophages (*right*); Grocott staining. **E** Residual lesion permeated by sparse collagen fibers; picrosirius staining. **F** Detail of the thin collagen structure of this lesion (*yellow fibers*) observed with polarized light. **G** Pseudoxantomatous macrophages in residual lesion; Masson's trichrome. **H** Degenerated yeasts of Pb18, within pseudoxantomatous macrophages in residual lesion; Grocott's staining. A/Sn mice, 1 month (**A–D**) and 4 months (**E–H**) after i.p. infection with 5×10^6 yeasts of Pb18. **A, E** ×250; **B, F** ×100; **C, D** ×125; **G, H** ×500
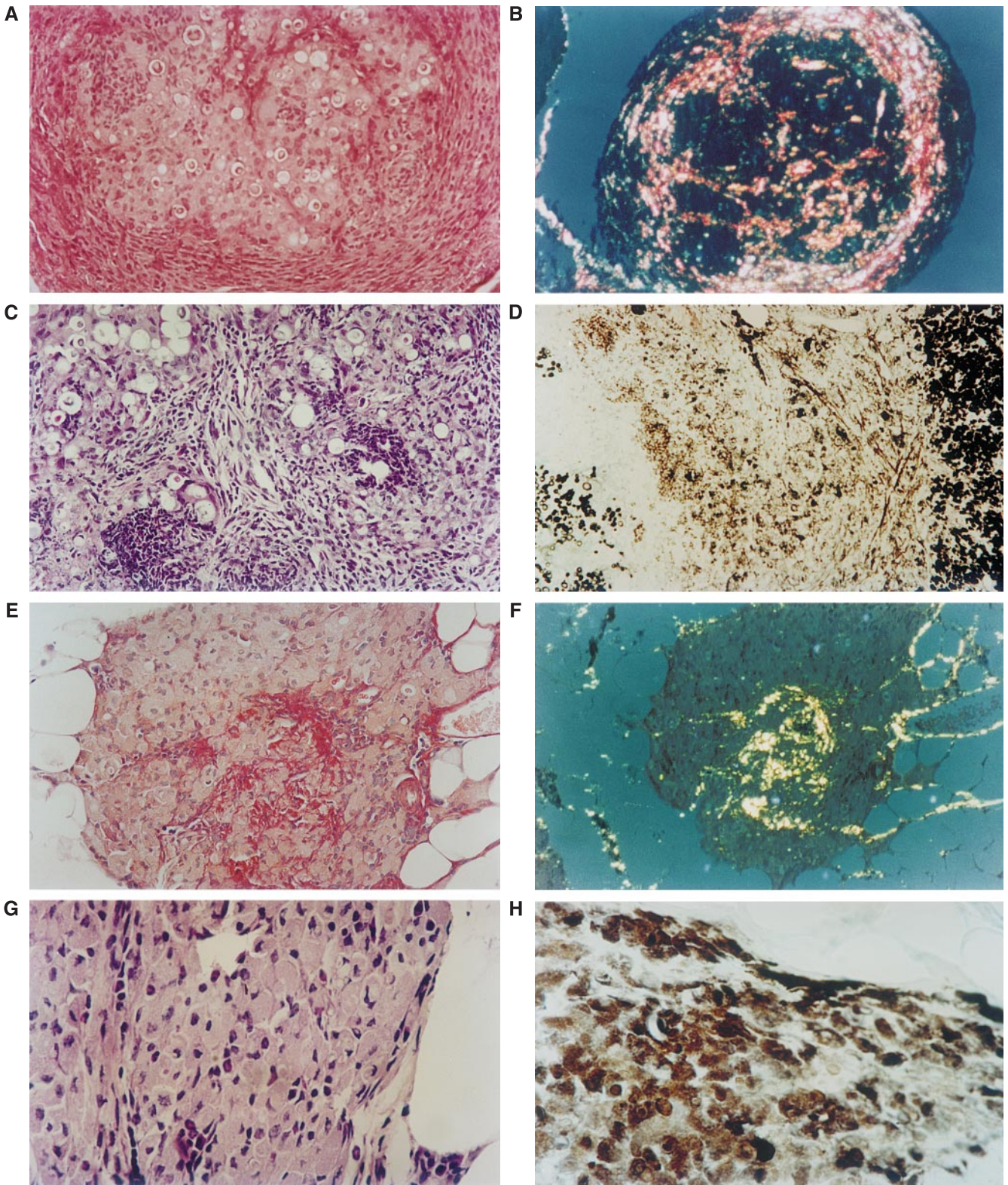
Comparison of paracoccidioidomycotic lesions evolution in resistant and susceptible

The characteristic patterns of lesions developed by each mouse strain are already well established by 3 months post infection. The striking differences in the number, type and size of the lesions observed in A/Sn and B10.A mice during these 3 months are indicated in Table 1. After 2 months the infection is under control in mice of the resistant strain, as shown by the
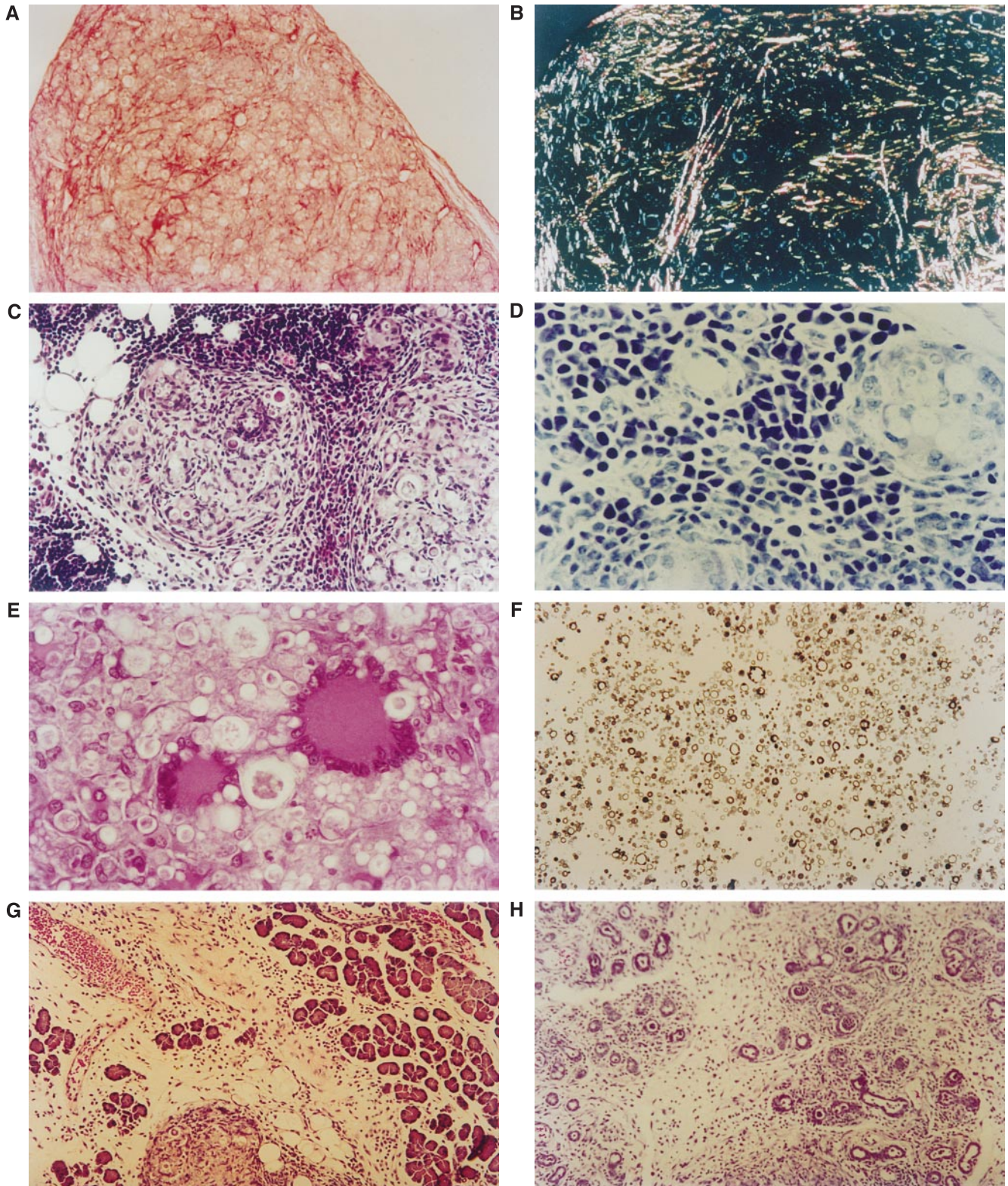
increasing percentage of residual lesions in which most of the fungi were degenerated. On the other hand all the lesions found in mice of the susceptible strain were active, containing fungi with well preserved morphology. Moreover, the mean size of the granulomas of B10.A mice at 3 month post infection is strikingly larger when compared to those found at the previous time points analyzed; in the subsequent months the sizes of the lesions tended to stabilize (data not shown).

Fig. 3 A Nodular non-capsulated peripancreatic lesion permeated by delicate collagen fibers; picrosirius staining. **B** Detail of the ECM proteins in a non-capsulated lesion showing collagen fibers (*yellow/green/red fibers*) and *P. brasiliensis* yeasts (*green spherical structures*); picrosirius staining plus polarized light. **C** Granulomatous foci surrounded by ECM compounds and mononuclear cells; H&E. **D** Plasmocytes in the periphery of the granulomatous foci; Giemsa stain. **E** Two Langhans' giant cells with many nuclei and PB18 yeasts; H&E. **F** Pb18 yeasts in granulomatous foci; Grocott's staining. **G** Discrete pancreatic lesion involving few peripheral pancreatic lobules; H&E. **H** Severe pancreatic lesion with destruction of many lobules; H&E (*ECM* extracellular matrix). **A, B, G, H** At 2 months after i.p. infection with 5×10^6 yeasts of Pb18; **C, D, E** At 1 month after infection; **F** At 4 months after infection in mouse strain B10.A (**A–F, H**) and A/Sn (**G**). **A, C, E–H** ×125; **B** ×200; **D** ×500

Discussion

The observed meager involvement of the pancreatic tissue in experimental murine PCM suggests that these lesions were established by contiguity from those present in the peripancreatic omentum. The present results, as well as the previous ones obtained in this model, show that the fungal lesions in abdominal organs were frequently restricted to their periphery, at their capsules. The connective tissue of the omenta drained the fungal inoculum, thus representing the target organ in the paracoccidioidomycotic i.p. infection. Our present results show that the pancreatic tissue of mice is mostly preserved after Pb18 i.p. infection, even in the susceptible B10.A strain. In a previous study of the kinetics of plasma amylase, marked differences between resistant and susceptible mice were demonstrated at 1 month post-infection, with an increase in the levels of this enzyme only being observed in susceptible B10.A [34]. The present histological findings are in accordance with these data, indicating that the early alteration detected in the exocrine function of this organ is transitory and reversible. The arrangement of the paracoccidioidomycotic lesions is very different in resistant and in susceptible mice. It is known that the deposition of the various components of the ECM is dependent on several factors. For instance, TGF-β promotes the recruiting, proliferation and activation of fibroblasts as well as the synthesis of collagen by these cells [33]. The presence of cytokines in the lesions has not yet been an object of study in experimental PCM; however, it has been shown that the production of IFN-γ by total lymph nodes cells is higher in resistant mice [4], and that of TGF-β tends to be higher in susceptible mice in an early phase after fungal i.p. infection (24 h) [25].

In resistant A/Sn mice we observed a massive lysis of *P. brasiliensis* yeasts in situ, denoted by the large amount of free and intracellular fungal detritus found in the granulomas; in contrast, in the susceptible B10.A strain the fungus survived, maintaining its morphology and actively budding, as detected progressively at later phases of the infection.

The overall cellular reaction developed by resistant mice culminated in the lysis of *P. brasiliensis*, whereas the one developed by susceptible mice allowed the survival of the fungi in the lesions. This shows that the cells involved are in a different state of activation. Indeed, it has previously been shown in the murine model of PCM that at the site of subcutaneous inoculation of *P. brasiliensis*, at 15 days post infection, the PMN from resistant mice are activated, whereas those from susceptible mice are not, as evaluated by the production of H_2O_2 and O_2^-. This activation was parallel to the fungicidal activity, so that the PMN of resistant mice killed *P. brasiliensis* in vitro and those of B10.A did not [24]. These data suggest that different cytokines and/or cell subsets may be present at the site of the lesion. Calich et al. [4] have recently demonstrated that there is preferential production of IFN-γ and IL-2, derived from type 1 T lymphocytes, during the entire course of infection in resistant mice but only a negligible amount of IFN-γ at the initial phases of the infection and no IL-2 in susceptible mice. These type 1 cytokines are important in stimulating phagocytosis by macrophages and in enhancing their antimicrobial activity [26].

From our present observations, there is a very strong indication that the PMN are responsible for the death of the fungi. The time-courses for presence of PMN and that for lysed yeasts are similar; moreover, the physical proximity of the fungi and the PMN strongly suggests that the role of this cell population is to effectively control the initial fungal load, by lysing *P. brasiliensis*. The macro-

Table 1 Evolution of pancreatic and peripancreatic lesions during the first 3 months after i.p. inoculation of resistant and susceptible mice with 5×10^6 yeast forms of *Paracoccidioides brasiliensis*

Mouse strain	Resistant (A/Sn)			Susceptible (B10.A)		
Months post-infection	1	2	3	1	2	3
Number of lesions[a]	39 (5)	20 (4)	19 (3)	65 (5)	119 (5)	112 (5)
% of active lesions[b]	92.3	40	5.3	100	100	100
% of residual lesions[b]	7.7	60	94.7	0	0	0
Mean size (μm^2)[c]	5166	1412	2901	2627	9285	27,009

[a] Sum of lesions found in pancreas and epiploon, with the number of analyzed mice in each time post-infection in parentheses
[b] Characteristics of the lesions, active or residual, as defined in Results

[c] Mean area of the lesions, calculated as described in Material and methods

phages, on the other hand, are most likely to be acting as scavenger cells, removing cellular and fungal debris originating by the action of PMN, in spite of the previous demonstration that macrophages from resistant mice are more activated that those from susceptible ones, as evaluated by H_2O_2 production, spreading activity and maintained expression of class II antigens [8]. The data reported here indicate distinct roles for different phagocytic cell populations in the evolution of the paracoccidioidomycotic granulomatous lesions. The PMN, present in large numbers in A/Sn mice, seem to be able to kill *P. brasiliensis*, whereas the macrophages from resistant mice, specially in residual lesions, have a foamy appearance resulting from fungi debris in their interior. This association between degenerated fungi and presence of PMN and pseudoxantomatous macrophages has already been described by Burger et al. [3], studying other model of susceptibility and resistance in athymic and euthymic BALB/c mice i.p. infected with *P. brasiliensis*.

The ECM deposition pattern on the lesions differed between the resistant and susceptible strains. In A/Sn mice the active granulomas containing viable yeasts were encapsulated, showed large quantities of dense collagen fibers, probably collagen type I, identified by the picrosirius stain plus polarized light as red fibers. In B10.A mice, the granulomatous lesions were sustained by a thin fibrotic structure which do not define a capsule, composed mostly of reticular collagen type III, in close contact with yeasts and macrophages. In the residual lesions of resistant A/Sn mice, with no viable *P. brasiliensis* yeasts, the ECM fibers were almost absent. Thus, the interaction between the antigens of the pathogen and host defenses result in a differential activation of the ECM.

In resistant A/Sn mice the cellular reaction is effective, promoting a marked pathogen destruction by activated neutrophils and the delimitation of the area of injury by ECM components. In susceptible B10.A mice, on the contrary, the fungal multiplication is not controlled, indicating inefficient activation of cellular mechanisms, thus permitting the thriving of *P. brasiliensis* and the impairment of its seclusion by ECM deposition. Therefore, the present work strongly suggests that the host's genetic pattern determines differences in the outcome of tissular reactions to *P. brasiliensis* i.p. infection.

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