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EXPERIMENTAL PULMONARY FIBROSIS INDUCED BY *PARACOCCIDIOIDES BRASILIENSIS* CONIDIA: MEASUREMENT OF LOCAL HOST RESPONSES

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Abstract. Pulmonary fibrosis was induced following inoculation of *Paracoccidioides brasiliensis* conidia intranasally in BALB/c mice. Fibrosis was associated with formation of granulomas, increase in lung hydroxyproline, and sustained increases in tissue tumor necrosis factor-α and transforming growth factor-β. This study suggests a role for these cytokines in generation of pulmonary fibrosis associated with chronic granulomatous infectious diseases.

Fibrosis is a frequent and incapacitating sequellae of numerous infections,[1–3] immune disease,[4] and exposure to toxic drugs.[5] It may also be the result of cryptogenic processes.[6–8] In these diseases, fibrosis shares common characteristics, including fibroblast proliferation and extracellular matrix deposition.[9–11]

Lung fibrosis often follows a series of chronic granulomatous processes. Although fibrosis is a frequent sequella, our knowledge of the various events leading to pulmonary damage is still scanty. Frequently, fibrosis begins with inflammation and leukocyte infiltration, followed by increased cytokine generation.[7–9] These reactions are thought to promote excess accumulation of connective tissue, which usually results in structural and functional alteration of the tissues involved.[9]

Based largely in vitro experiments, tranforming growth factor-β (TGF-β), tumor necrosis factor-α (TNF-α), interleukin-1 (IL-1), and IL-6 have all been implicated in the generation of fibrosis (Table 1).[12–16] Results of in vivo studies have in some cases appeared to conflict. The apparent conflicts have occurred with different investigator's studies. Cytokine responses in different diseases and experimental conditions have been conflicting, showing stimulation or suppression with the same cytokines. However, the in vivo studies have been limited.[12, 17, 18] This may be a reflection of the difficulties existing for the establishment of progressive fibrosis in experimental animals.[19, 20] We have tried unsuccessfully to establish pulmonary fibrosis using mouse models of systemic mycoses. In our experience, coccidioidomycosis in BALB/c mice has been a lethal infection. In unpublished studies we have found that pulmonary *Histoplasma capsulatum* infection in ICR or BALB/c mice with yeast forms or conidia is followed either by death or complete recovery. Cryptococcosis is not associated with fibrosis. Therefore, we turned to paracoccidioidomycosis.

Paracoccidioidomycosis is the most important fungal disease in some areas of Latin America.[3, 21] This mycosis is a chronic, progressive disease caused by the thermally dimorphic fungus *Paracoccidioides brasiliensis*. Primary infection occurs in the lungs, where it causes chronic damage of the parenchyma leading to fibrosis and severe restriction of respiratory function.[3, 21, 22] Such pathology is observed in as many as 80% of patients with this disease.[3, 21, 23] We have developed a model of pulmonary fibrosis in BALB/c mice induced by the intranasal infection of *P. brasiliensis* conidia.[24] This model has allowed us to evaluate pulmonary tissue responses occurring during the active and residual processes. The present study was undertaken to determine whether fibrosis is associated with alterations in local immune mediators during the course of experimental pulmonary paracoccidioidomycosis.

MATERIALS AND METHODS

Animals. Adult (6–8 weeks old) male and female BALB/c mice were obtained from our specific pathogen- free breeding colony and maintained under barrier conditions by the Research Service of the Audie L. Murphy Memorial Veterans Hospital (San Antonio, TX). This is an National Institutes of Health approved animal care facility and meets humane guidelines for care of laboratory animals.

Inoculum. The *P. brasiliensis* American Type Culture Collection (Rockville, MD) 60855 isolate, known to produce abundant conidia,[11] was used in all experiments. For production of conidia, the mycelial phase of the fungus was grown on water-agar plates (10 g of Bacto agar [Difco, Detroit, MI] per 1,000 ml of distilled water) and then incubated at 18°C for eight weeks.[25] For each experiment, the infectious conidia (propagules) were collected as follows. The plates on which *P. brasiliensis* cultures were grown in the mycelial phase were flooded with physiologic saline with added containing 0.85% Tween 20. The growth was scraped from the agar surface with a gauged loop. The suspension was transferred to a capped Erlenmeyer flask containing three layers of 6-mm glass beads. The flask was then shaken on a gyrorotatory machine for 30 min at 250 revolutions/min. to free the conidia. The fungal slurry was then poured through a sterile syringe packed with glass wool. The conidia suspension that passed through the glass wool were concentrated by centrifugation and the supernatant was decanted. The sediment, which was rich in conidia, was suspended in 50 ml of physiologic saline and agitated vigorously to suspend the conidia. The number and viability were determined using staining with ethidium bromide.[26] The viability of the conidia was consistently higher than 95% of total number of conidia counted. The inoculum was adjusted so that 0.06 ml contained approximately 3×10^6 viable conidia.[27]

Experimental infection. Animals were anesthetized by intramuscular injection of a solution containing 3 mg of ketamine hydrochloride (Bristol Laboratories, Syracuse, NY), 0.4 mg of Xylazine® (Phoenix Pharmaceuticals, St. Joseph, MO) plus 0.1 ml of saline per 25 g of body weight. When deep anesthesia was obtained, 3×10^6 conidia (in 0.06 ml

TABLE 1
Actions of various cytokines in relation to fibrosis

Cytokine	Stimulation of fibrogenesis	Inhibition of fibrogenesis	References	Year
Interleukin-1	Yes	Yes	12	1990
			13	1991
			14	1994
			16	1995
Tumor necrosis factor-α	Yes	Yes	12	1990
			13	1991
			14	1994
			16	1995
Transforming growth factor-β	Yes	Yes	13	1991
			14	1994
			16	1995
Platelet-derived growth factor	Yes		13	1991
			14	1994
Epidermal growth factor	Yes		13	1994
Interferon-γ	Yes	Yes	12	1990
			13	1991
			14	1994
Interleukin-4	Yes		14	1994
			15	1992
Interleukin-6	Yes		14	1994
Insulin-like growth factor	Yes		14	1994
Endothelin-1			14	1994
Fibroblast growth factor			14	1994

of the inoculum) were instilled intranasally. The abdomen was compressed, and a droplet was deposited on the nares. When the pressure was released, the mouse inhaled deeply.

Control mice received an intranasal inoculum of 0.06 ml of saline. To control for changes in collagen and cytokines during aging, each group of infected mice was studied with age-matched controls housed in the same conditions as infected mice.

Animals were killed by barbiturate overdose injected intraperitoneally. To avoid errors in measuring lung weights, different mice were used for studies of bronchoalveolar lavage (BAL) and lung homogenates. Because we wished to 1) have a cumulative six animals studied per time point, 2) study multiple parameters per time point, and 3) include age-matched controls for hydroxyproline, we conducted multiple studies using similar inoculum and post infection care. These studies are combined in the results, which show cumulative data on all mice at 1, 2, and 3 days and at 1, 2, 4, 8, 12, and 16 weeks after infection. Infection with *P. brasiliensis* progresses slowly, with no mortality until weeks 10–12, at which time about 25% of the mice die. The data obtained on mice 12 and 16 weeks after infection were obtained from surviving mice. By week 20, 50–70% of mice die of infection. Because of limited isolator space, and because of differences in tissue processing, separate studies were done for histopathology (34 mice), quantitation of tissue cultures (54 mice), measurement of hydroxyproline (72 mice), and measurement of cytokines in BAL and in lung homogenates (240 mice).

Quantitative cultures. Lungs were removed from mice under aseptic conditions, weighed, and homogenized in 2 ml of saline; the lung tissue homogenate was serially diluted and plated in duplicate on Sabouraud's agar. After 30 days of incubation at 18°C, the number of colony-forming units (CFU)/g of the tissue was calculated.

Histopathology. The lungs were resected and fixed in 10% phosphate-buffered formalin. Some lungs were expanded by intratracheal instillation of formalin to a water pressure of 20 cm. Histopathologic sections were stained with hematoxylin and eosin and used to study the characteristics of the inflammatory response. Gomori's silver reticulin–stained sections were used to confirm the changes occurring in the organization of reticulin fibers (collagen III), and Masson's trichrome–stained preparations were used to identify collagen I fibers. To determine the severity of lung involvement with fibrosis, we used the same parameters previously reported.[24] The scoring method is summarized in Table 2.

Hydroxyproline determination. Total lung content was determined by assaying for hydroxyproline concentrations after hydrolysis with 6 N HCl. Lungs were removed and the trachea and main stem bronchi were dissected free and discarded. The lungs were then homogenized at a 1:60 dilution in 0.25 M sucrose. A 0.5-ml sample was withdrawn and added to 0.5 ml of 12 N HCl, vortexed, and digested overnight in a sealed test tube at 120°C. All samples were run in duplicate and the results were averaged. The samples were processed for determination of hydroxyproline as described by Woessner.[28]

Cytokine assays. *Bronchoalveolar lavage.* After mice were killed, the trachea was exposed and a blunt tipped needle was inserted into the trachea. One milliliter of RPMI 1640 medium was then flushed into the lungs once and aspirated. Bloody fluids were discarded. Fluids were frozen at −70°C until assayed.

Lung homogenates. After mice were killed, the lungs were removed, individually homogenized in 2 ml of sterile saline, and aliquots of the homogenate were frozen at −70°C until assayed. Aliquots of these samples were thawed only once immediately prior to performing cytokine assays. The assays used were a commercial ELISA for murine cytokines (TNF-α; Endogen Inc., Boston, MA and TGF-β; Genzyme Corp., Cambridge, MA).

Statistical analysis. Data are expressed as the mean ±

TABLE 2
Histopathologic scoring of pulmonary fibrosis[24]*

	Mild	Severe
Hematoxylin and eosin	Small inflammatory foci, <50% alveoli	>50% alveoli plus adjacent bronchi involved
Trichrome stain	Isolated fibers in and around inflammatory foci	Abundant thick fibers about inflammatory foci
Reticulin stain	Fragmentation, reorganization, condensation of fibers in/around foci	Increased numbers fibers and changes in location of fibers in and around inflammatory foci

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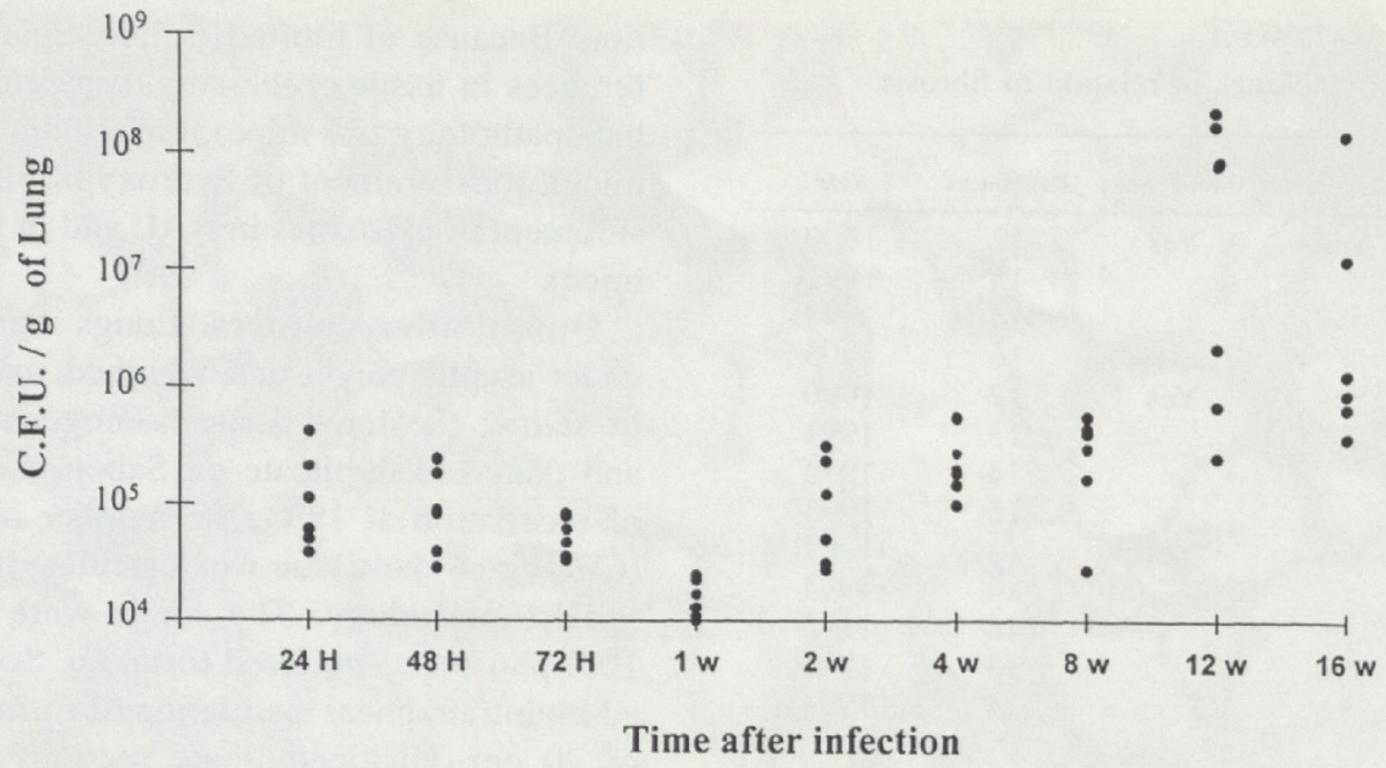

FIGURE 1. *Paracoccidioides* tissue counts in the lungs of BALB/c mice infected with *P. brasiliensis* conidia. ● = individual counts; C.F.U. = colony-forming units; H = hours; w = weeks.

standard error. Uninfected controls and infected animals were compared using one-way analysis of variance. Results were considered significantly different for P values < 0.05. The actual P values as given for most comparisons with significant differences were < 0.0001. The calculations were done using Stathagraphics Plus, Version 6.2, 1991 (Statistical Graphics Corporation, Princeton, NJ). The multiple range analysis test for significant differences was used compare concentrations of cytokines in BAL and lung tissue of infected mice with those in control mice.

RESULTS

Quantitative preliminary cultures. As shown in Figure 1, the intranasal challenge with *P. brasiliensis* conidia produced a chronic infection. The fungus was isolated throughout the course of the experiments. The number of CFU decreased during the first week and slowly increased through the 12th week ($P < 0.001$).

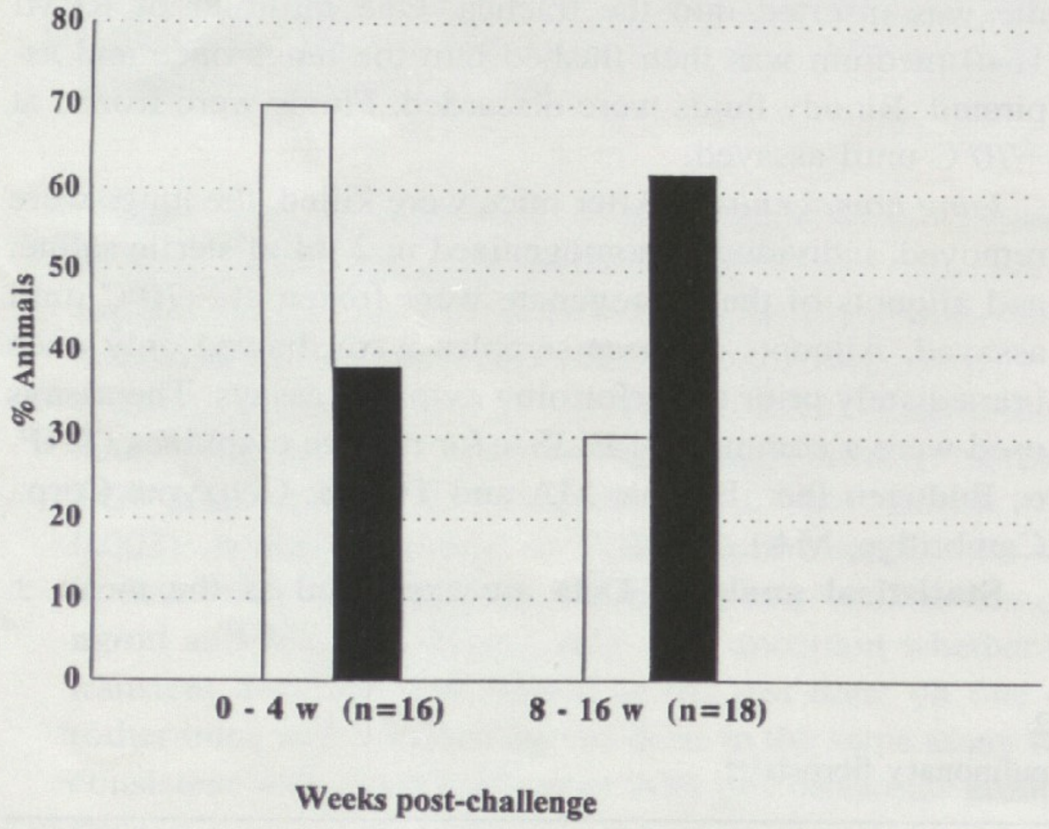

FIGURE 2. Histologic determination of fibrosis in BALB/c mice based on increase and/or dearrangement of interstitial connective tissue using Masson's trichrome stain for collagen I and reticulin stain for collagen III. Multiple sections of lungs of each mouse were examined. Light bars = mild involvement; dark bars = severe involvement. w = weeks.

Histopathology. The sequential analyses of the histopathologic changes in 34 mice studied indicated that the development of fibrosis took place gradually (Figure 2). Up to four weeks, fibrosis was minimal in 70% of the animals. From eight through 16 weeks, severe fibrosis was present in 60% of the mice. Lung sections at four weeks postinfection showed no major change in collagen (Figure 3A) or in the organization of the reticulin fibers (Figure 3B). However, by 12 weeks after infection large areas of the lungs had became consolidated. There were numerous fungal cells and a marked increase in collagen I (Figure 3C), as well as fragmentation and reorganization of reticulin fibers (collagen III) (Figure 3D). Granulomas when seen were identical in appearance to those seen in human paracoccidioidomycosis. Inflammatory foci were initially seen located around bronchi and blood vessels, but were not obstructing them or causing obvious ischemia.

Hydroxyproline concentration. In comparison with noninfected controls, infected mice had increased concentrations of hydroxyproline by eight weeks postchallenge, ($P = 0.0237$) (Figure 4). Mice at weeks eight, 12, and 16 had much higher hydroxyproline concentrations than mice at one, two, and four weeks ($P < 0.006$). At weeks 12 and 16, the concentrations of hydroxyproline were 3.6 and 8.6 times higher than in control animals of the same age.

Cytokine determination. *Tumor necrosis factor-α.* **Bronchoalveolar lavage.** As shown in Figure 5, a marginally significant increase in infected mice over the control values was noticed only at days one and two postchallenge. Subsequently, TNF-α was not detectable for the remaining observations.

Lung homogenates. In contrast with BAL, TNF-α levels were significantly higher in homogenates from infected animals than in the six controls in which TNF-α was not detected throughout the observation period ($P = 0.0003$). The maximal levels were detected at two weeks postchallenge. At eight, 12, and 16 weeks, the TNF-α concentrations were lower than in earlier weeks, but still remained above control values ($P = 0.0013$) (Figure 5).

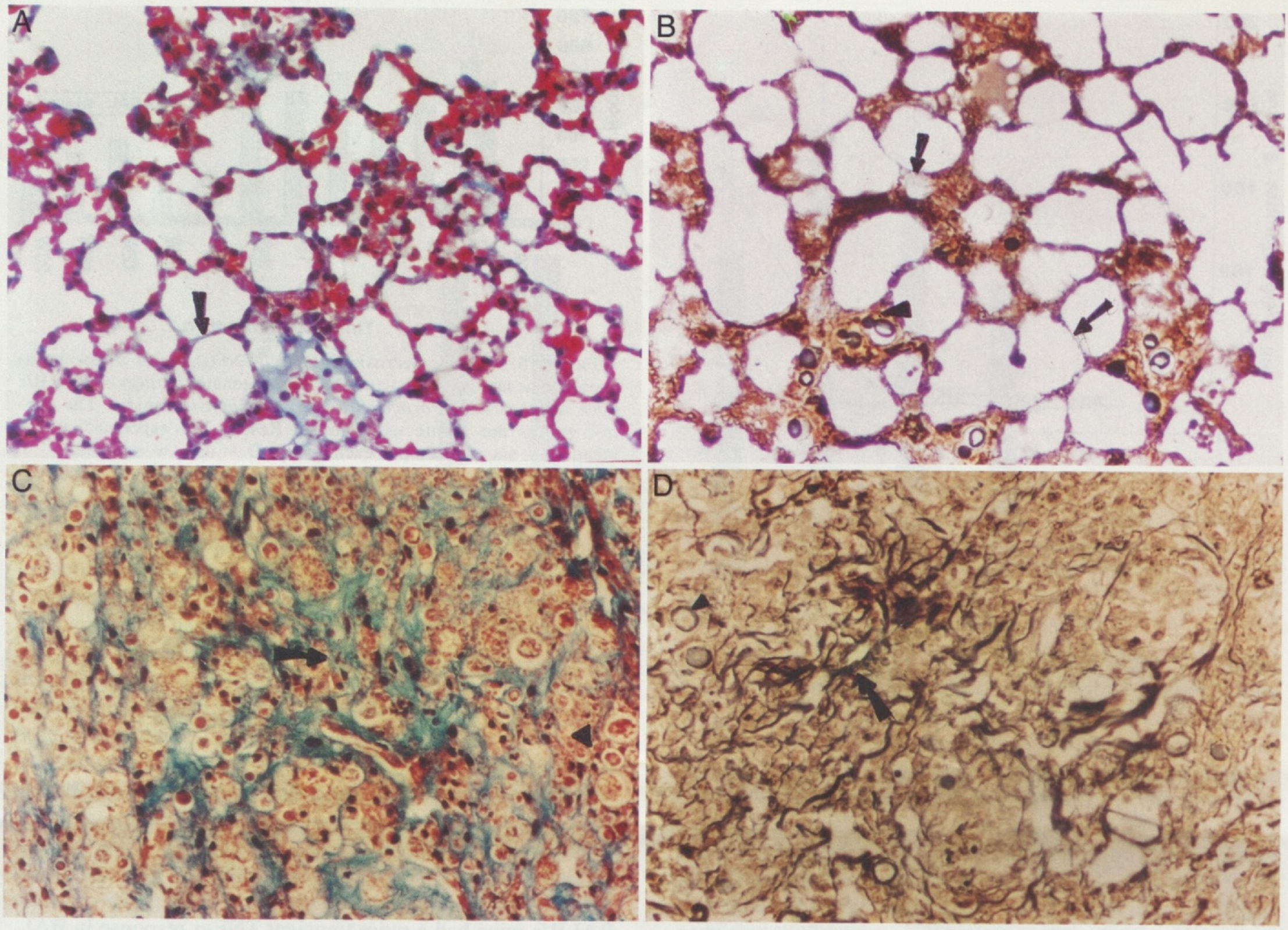

FIGURE 3. Histopathology in BALB/c mice induced by infection with *Paracoccidioides brasiliensis*. **A,** minor involvement four weeks postchallenge. Thin collagen I fibers are seen in the alveolar wall **(arrow)** (Masson's trichrome stained). **B,** minor involvement four weeks post-challenge. Thin collagen III fibers are seen in the alveolar wall **(arrows).** The **arrowhead** indicates a *P. brasiliensis* yeast (Gomori's silver stained). **C,** severe involvement 12 weeks postchallenge. Thick collagen I fibers that form bands **(arrow)** and abundant *P. brasiliensis* yeast **(arrowhead)** are seen (Masson's trichrome stained). **D,** severe involvement 12 weeks postchallenge (*P. brasiliensis* is shown at the **arrowhead**). There is an increase in collagen III fibers, which appear crossing and facing each other **(arrow)** (Gomori's silver stained). (Original magnification × 400.)

Transforming growth factor-β. **Bronchoalveolar lavage.** In both control and experimental animals and with one exception (week 16), this factor was not detected in the BALs tested during various times after infection (Figure 6).

Lung homogenates. In contrast to BAL, lung homogenates from infected mice exhibited significantly increased TGF-β concentrations when compared with controls ($P < 0.003$). A sharp decrease in TGF-β at two weeks was seen in four mice (< 1 ng/ml), while two mice had levels of 3 ng/ml and 8 ng/ml, respectively. It is uncertain whether this transient decrease was related to the test done on one day (other mice and standard curves done in the same assay were consistent with prior studies) or with this particular group of mice.

DISCUSSION

The isolation of *P. brasiliensis* from the lungs at all times during the experimental period clearly indicates that a persistent, progressive infection was established. Counts of *P. brasiliensis* decreased during the first week after infection but then steadily increased through the end point of the study at 16 weeks, being highest at 12 and 16 weeks. The late stage of infection was also associated with increasing mortality.

Sequential histopathologic observations revealed, as previously demonstrated,[24] the gradual development of pulmonary fibrosis beginning at eight weeks and culminating at 12–16 weeks postinoculation when consolidation of the tissues damaged by fungal infection was observed and probably contributed to mortality. Increasing fibrotic damage in and around the site of fungal infection has also been seen in histopathologic studies and autopsies of patients with paracoccidioidomycosis.[3, 22, 29–31] The fibrotic sequellae appeared in every organ or site infected. Hydroxylation of proline to hydroxyproline is one of the main steps in collagen synthesis, and the measurement of the hydroxyproline content of tissues is a rather direct measurement of the extent of col-

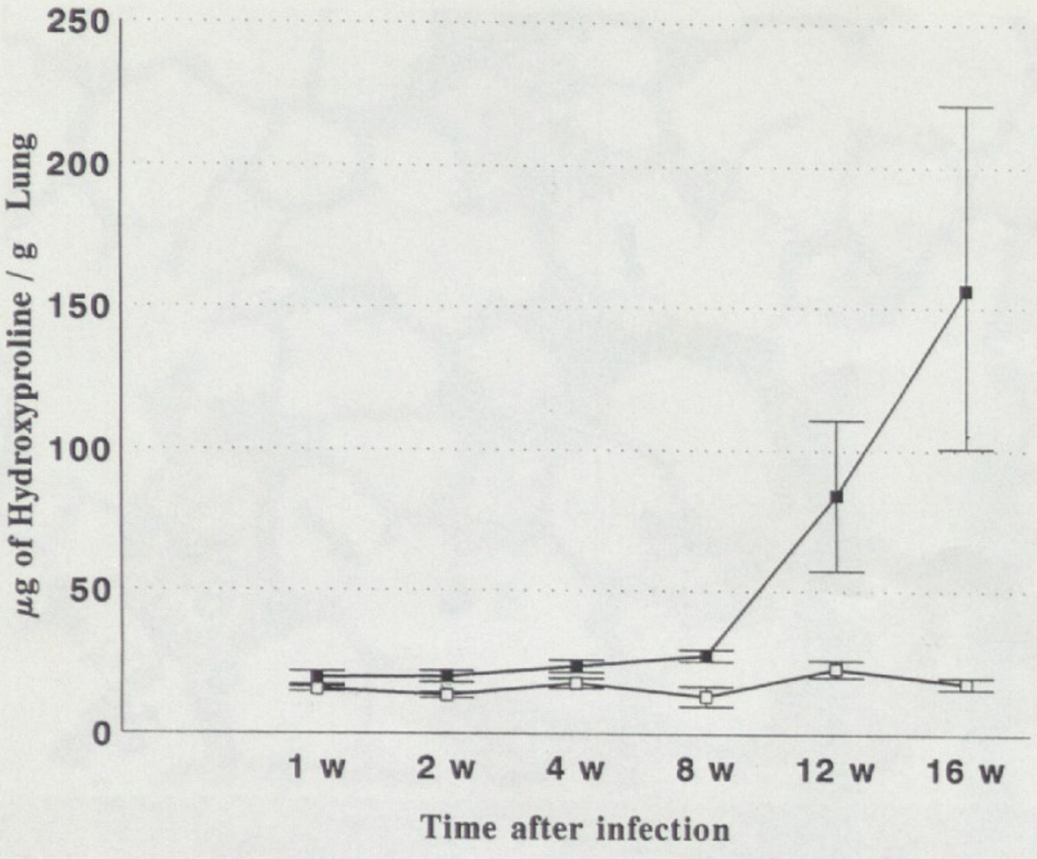

FIGURE 4. Hydroxyproline levels in the lungs of BALB/c mice during the course of paracoccidioidomycosis. □ = controls; ■ = infected. Results are expressed as the mean ± SE. n = six animals per group. w = weeks.

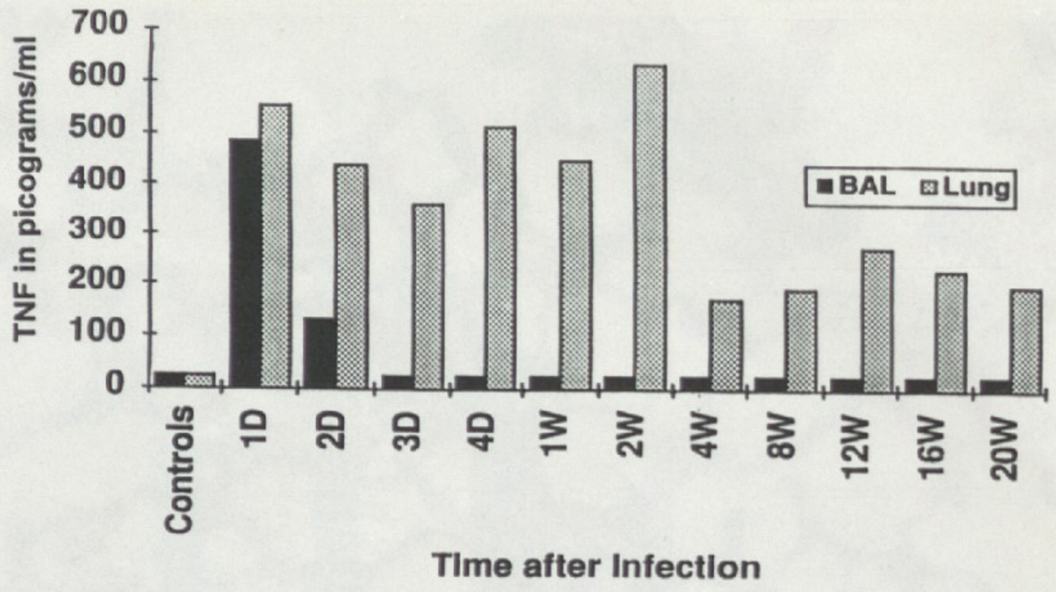

FIGURE 5. Tumor necrosis factor-α (TNFα) levels in the bronchoalveolar lavage (BAL) and lung homogenates (Lung) of BALB/c mice infected with *Paracoccidioides brasiliensis* conidia. The control groups are uninfected animals. Results are expressed as the mean. n = six animals per each group. D = day; w = weeks.

lagen, and indirectly fibrosis. The histopathologic studies and increased hydroxyproline concentration measured as a component of dry lungs do not support edema as a significant component of increased lung mass.

Paracoccidioidomycosis is at present the only infectious animal model of progressive pulmonary fibrosis. Fibrosis was initially observed at eight weeks after infection, a time when hydroxyproline levels had modestly increased in lungs of infected animals. A much sharper increase occurred at 12 and 16 weeks. Assay of hydroxyproline levels thus allowed a more quantitative assessment of density of fibrosis than histopathology, which described local disease rather than the overall process.

Macrophages and other cells when appropriately stimulated may produce a variety of cytokines and chemokines. In the present studies, we concentrated on just two of these, TNF-α and TGF-β because of prior reports of their association with pulmonary fibrosis. Both TNF-α and TGF-β were undetectable in BAL and lung homogenates of uninfected control mice. Within 24 hr after infection, there was a sharp increase in TNF-α levels in both lung homogenates and BAL, and in TGF-β in lung homogenates. As infection proceeded, TNF-α rapidly decreased in BAL, but persisted at elevated concentrations in the lung homogenates, as did TGF-β. Levels of TGF-β persisted at high concentrations throughout infection, while levels of TNF-α slowly decreased, but not to the undetectable levels of controls. Thus, the present studies show a correlation, though not necessarily causality, between lung tissue TNF-α and TGF-β and the development of fibrosis. It is not clear whether the prompt increase in TNF-α contributed to fibrosis, or whether a more chronic and sustained elevation was more significant. In other studies, Piguet and others found that bleomycin-induced fibrosis was associated with increases in TNF-α and that antibody to TNF-α abrogates the fibrosis.[18] These data support a causal role for TNF-α in pulmonary fibrosis caused by bleomycin.

In clinical practice, others have suggested that BAL provides an appropriate means for evaluating the pulmonary immune response.[32] However, the present studies of paracoccidioidomycosis and the independent observations of Phan and others[33] and Schreir and others[34] (with bleomycin) suggest that cytokine responses of tissues are associated more with evolving fibrosis than BAL. In the subacute bleomycin model of Phan and others,[33] CBA/J mice developed fibrosis after being treated with bleomycin, while BALB/c mice had no fibrosis. In spite of the fact that we also used BALB/c mice, which developed fibrosis, direct comparisons are difficult because of the different stimulus for fibrosis, and because Phan and others used cytotoxicity assays for TNF-α and we used an ELISA. The CBA/J mice had increasing lung interstitial cell TNF-α activity to four weeks after treatment, and a late increase in BAL TNF-α, while BALB/c mice had a more transient TNF-α response in BAL, and no response in interstitial cells. This may reflect that a single dose of bleomycin was used or potential interference with cytokine secretion caused by collagen deposition. In a detailed review, Kumar and Lykker hypothesize that cells may require physical contact to transmit messages of fibrotic mediators.[32]

It has been suggested that the fibrotic response to pulmonary injury is reduced in T cell–deficient animals.[34] However, other studies indicated that fibrosis can progress in athymic mice (Morris MJ and others, unpublished data). Athymic mice after adoptive transfer of Chinese hamster ovary cells producing TNF-α developed progressive fibrosis, whereas control Chinese hamster ovary cells, which did not produce TNF-α, did not cause fibrosis. Unlike our studies of paracoccidioidomycosis, these tumor cells were not regulated by any biological feedback mechanism, and it is possible that a more dynamic infectious process such as paracoccidioidomycosis might impair the cytokine network or lymphocyte/macrophage traffic through fibrotic tissue as disease progresses.

The absence of detectable TGF-β in controls and its sustained increase in lung homogenates after infection also suggest that this cytokine may play a role in the generation of fibrosis.[14, 35, 36] However, there are at present no studies indicating that bleomycin-induced fibrosis can be abrogated by antibody to TGF-β.

In summary, the present model is the first infectious ani-

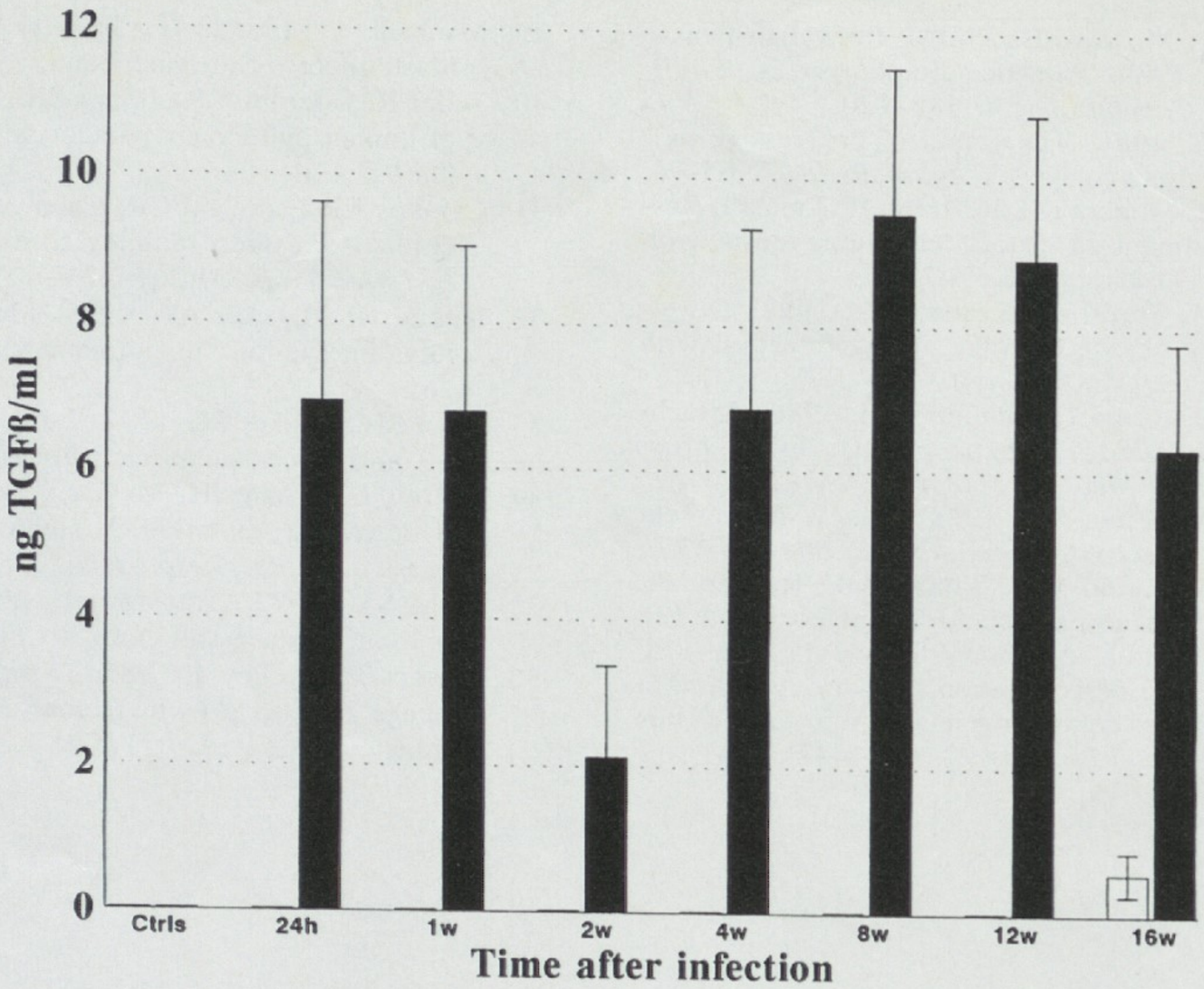

FIGURE 6. Transforming growth factor-β (TGF-β) levels in bronchoalveolar lavage (light bars) and lung homogenates (dark bars) of BALB/c mice infected with *Paracoccidioides brasiliensis* conidia. The control groups are uninfected animals. Results are expressed as the mean ± SE. n = six animals per each group. Ctrls = controls; h = hours; w = weeks.

mal model of chronic progressive pulmonary fibrosis. Fibrosis is associated with sustained increases in lung tissue levels of TNF-α and TGF-β. Neutralization studies using this model should permit a determination of whether either or both of these cytokines are merely associated with progressive fibrosis, or whether they play direct causal roles.

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REFERENCES

1. Scott PA, Sher A, 1993. Immunoparasitology. Paul WE, ed. *Fundamental Parasitology.* Third edition. New York: Raven Press, 1179–1210.
2. Goodwin RA, Nickell JA, Dez Prez RM, 1972. Mediastinal fibrosis complicating healed primary histoplasmosis and tuberculosis. *Medicine 51:* 227–232.
3. Brummer E, Castaneda E, Restrepo A, 1993. Paracoccidioidomycosis: an update. *Clin Microbiol 6:* 89–117.
4. Perez MI, Kohn S, 1993. Systemic sclerosis. *J Am Acad Dermatol 28:* 525–547.
5. Thrall RS, McCormick RM Jr, McReynolds RA, Ward PA, 1979. Bleomycin induced pulmonary fibrosis in the rat: inhibition by indomethacin. *Am J Pathol 95:* 117–127.
6. Cantin AM, Boilean R, Begin R, 1988. Increased procollagen III amino terminal peptide-related antigens and fibroblast growth signals in the lung of patients with idiopathic pulmonary fibrosis. *Am Rev Respir Dis 137:* 572–579.
7. Crystal R, Bitterman P, Rennard SI, Hance AK, Keogh BA, 1984. Interstitial lung disease of unknown cause. Disorder characterized by chronic inflammation of the lower respiratory (first part). *N Engl J Med 310:* 154–166.
8. Crystal R, Bitterman P, Rennard SI, Hance AK, Keogh BA, 1984. Interstitial lung disease of unknown cause. Disorder characterized by chronic inflammation of the lower respiratory tract (second part). *N Engl J Med 310:* 235–244.
9. Fridman SL, 1993. The cellular basis of hepatic fibrosis. Mechanisms and treatment strategies. *N Engl J Med 355:* 1835.
10. Bitterman PB, Rennard SI, Hunninghake G, Crystal RG, 1982. Human alveolar macrophase growth factor for fibroblasts. Regulation and partial characterization. *J Clin Invest 70:* 806–822.
11. Bitterman PB, Adelberg S, Crystal RG, 1983. Mechanisms of pulmonary fibrosis: spontaneous release of alveolar macrophage derived growth factor in the interstitial lung disorders. *J Clin Invest 72:* 1810–1813.
12. Elias JA, Freundlich B, Kern J, Rosenbloom J, 1990. Cytokine networks in the regulation of inflammation and fibrosis in the lung. *Chest 97:* 1439–1445.
13. Kovacs EJ, 1991. Fibrogenic cytokines: the role of the immune mediators in the development of scar tissue. *Immunol Today 12:* 17–23.
14. Maquart FX, Gillery P, Borel JP, 1994. Cytokines and fibrosis. *Eur J Dermatol 4:* 91–97.
15. Postlethwaite AE, Holness MA, Katai H, Raghow R, 1992. Human fibroblasts synthesize elevated levels of extracellular matrix proteins in response to interleukin-4. *J Clin Invest 90:* 1479–1485.
16. Hunninghake GW, Kalica AR, 1995. Approaches to the treatment pulmonary fibrosis. *Am J Crit Care Med 151:* 915–918.
17. Gosset P, Perez T, Lasalle B, Duquesnoy B, Farre JM, Tonnel AB, Capron A, 1991. *Increased TNF alpha secretion by alveolar macrophages from patients with rheumatoid arthritis. Am Rev Respir Dis 143:* 593–599.
18. Piguet PF, Collart MA, Grau CE, Kapanci Y, Vassalli P, 1989. Tumor necrosis factor/cachectin plays a key role in bleomycin-induced pneumopathy and fibrosis. *J Exp Med 170:* 655–663.
19. Graybill JR, Ahrens J, Suchyta MR, Coalson J, 1988. Experimental pulmonary histoplasmosis and emphysema. *Am Rev Respir Dis 137:* 1193–1197.
20. Williams DM, Magee DM, Bonewald LF, Smith JG, Byrne GI, Schachter JA, 1990. Role in vivo for TNF alpha in host defense against *C. trachomatis. Infect Immun 58:* 1572–1577.

21. Franco MF, Mendez RP, Moscard-Bacchi M, Rezkaliah-Iwasso M, Montenegro MR, 1989. Paracoccidioidomycosis. *Bailliere's Clin Trop Med Commun Dis 4:* 185–220.
22. Londero AT, Severo LC, 1981. The gamut of progressive pulmonary paracoccidioidomycosis. *Mycopathologia 75:* 65–74.
23. Naranjo MS, Trujillo M, Munera MI, Restrepo P, Gomez I, Restrepo A, 1990. Treatment of paracoccidioidomycosis with itraconazole. *J Med Vet Mycol 55:* 67–76.
24. Restrepo S, Tobon AM, Trujillo J, Restrepo A, 1992. Development of pulmonary fibrosis in mice during infection with *P. brasiliensis* conidia. *J Vet Mycol 30:* 173–184.
25. Restrepo A, Salazar ME, Cano LE, Patino MM, 1986. A technique to collect and dislodge conidia produce by *P. brasiliensis* mycelial form. *J Med Vet Mycol 24:* 247–250.
26. Calich VL, Purchino A, Paula CR, 1978. A new fluorescent viability test for fungal cells. *Mycopathologia 66:* 175–177.
27. McEwen JG, Bedoya V, Patino MM, Salazar ME, Restrepo A, 1987. Experimental murine paracoccidioidomycosis induced by the inhalation of conidia. *J Med Vet Mycol 25:* 165–175.
28. Woessner JF Jr, 1961. The determination of hydroxyproline in tissue and protein samples containing small proportions of this amino acid. *Arch Biochem Biophys 93:* 440–447.
29. Machado J, Miranda JL, Teixeira GA, 1965. Das sequelas da blastomicos e Sul-Americana. *Hospital 68:* 141–147.
30. Tuder RM, Ibrahim R, Godoy CE, de Brito T, 1985. Pathology of human pulmonary paracoccidioidomycosis. *Mycopathologia 92:* 179–188.
31. Campos EP, Padovani CR, Cataneo AMJ, 1991. Paracoccidioidomicose: estudo radiologico e pulmonar de 58 casos. *Rev Inst Med Trop 33:* 267–276.
32. Kumar RK, Lykker AW, 1995. Messages and handshakes: cellular interactions in pulmonary fibrosis. *Pathology 27:* 18–26.
33. Phan SH, Kunkel SL, 1992. Lung cytokine production in bleomycin-induced pulmonary fibrosis. *Exp Lung Res 18:* 29–43.
34. Schreir DJ, Phan SH, McGarry BM, 1983. The effects of the nude (nu/nu) mutation on bleomycin produced pulmonary fibrosis. *Am Rev Respir Dis 127:* 614–617.
35. Wahl SM, 1992. Transforming growth factor beta in inflammation: a cause and cure. *J Clin Immunol 12:* 61–74.
36. Kovacs EJ, Kelley J, 1985. Lymphokine regulation of macrophage derived growth factor secretion following pulmonary injury. *Am J Pathol 121:* 261–268.