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IN VIVO PULMONARY RESPONSE TO *ASPERGILLUS TERREUS* SPORES*

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Abstract—The fate of intratracheally instilled *Aspergillus terreus* spores was followed in both rabbits and rats. Phagocytosis of the spores by the pulmonary macrophage was rapid in that approx. 42% of the observed spores were associated with the macrophages immediately after instillation. Direct penetration of the lung architecture by the spores was not observed but spores were seen in the alveolar interstitium at 3 hr after instillation and in the tracheobroncheal lymph nodes at 24 hr. Granulomas formed between 48 hr and 1 week after exposure. In the absence of apparent spore or spore extract toxicity and precipitating antibodies against *Aspergillus terreus*, the observed reactions preclude the possibility that the lesions were the consequence of hypersensitivity. This model of pulmonary response to fungal spores may be of future value for characterizing further the pathology associated with certain occupational exposures to moldy materials.

Key words: Aspergillus, lung, pathology, rabbits, rats

RESPONSE PULMONAIRE *IN VIVO* AUX SPORES *ASPERGILLUS TERREUS*

Résumé—L'action de l'instillation intra-trachéale des spores d'*Aspergillus terreus* a été suivie à la fois sur des lapins et sur des rats. La phagocytose des spores par le macrophage pulmonaire a été rapide en ce sens qu'approximativement 42% des spores observées étaient associées aux macrophages immédiatement après l'instillation. La pénétration directe des spores dans l'architecture du poumon n'a pas été observée mais on a pu voir des spores dans l'interstice alvéolaire 3 hr après l'instillation et dans les nodosités de la lymphe trachéobronchiale, 24 hr après. Des granulomes se sont formés de 48 hr à une semaine après exposition. En l'absence de toxicité des spores apparentes ou de celle d'extraits de spores ainsi que d'anticorps qui précipitent contre l'*Aspergillus terreus*, les réactions observées excluent la possibilité que les lésions aient été la conséquence d'une hypersensibilité. Ce modèle de réponse pulmonaire aux spores fongaux peut être valable dans le futur pour aller plus loin dans la caractérisation de la pathologie associée à certaines professions exposées à des matières en moisissure.

Mots-clefs: Aspergillus, poumon, pathologie, lapins, rats

INTRODUCTION

Fungi of the genus *Aspergillus* are ubiquitous in nature, occurring in association with decaying organic matter. They have been implicated in the pathogenesis of a number of pulmonary diseases including asthma and pulmonary eosinophilia, hypersensitivity pneumonitis, aspergilloma, and invasive aspergillosis [1]. Occupational as well as avocational

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exposures to *Aspergillus* spores have been recorded for many organic dusts including compost [2, 3], flour [4], grain dusts [5], and malt [6].

The mechanisms whereby fungi produce pulmonary disease in susceptible individuals are not fully understood. In the case of hypersensitivity penumonitis, the history of prior exposure, the time course of the reaction, and the presence of precipitating antibodies in the sera of the majority of patients suggest an allergic basis. However, despite a wealth of human and experimental data, it has not yet been possible to demonstrate a causal relationship between the abnormalities in cellular and humoral immune function and the disease. Other mechanisms, such as direct complement activation [7], platelet aggregation [8], or histamine release [9], either separately or in combination, may be involved. Previously reported studies from these laboratories have shown that the direct activation of complement by aerosols of *Aspergillus terreus* spores may lead to an acute arterial hypoxia in exposed rabbits [7, 8]. The rapid onset of the reactions together with the absence of pathological changes in the lungs of exposed animals led us to speculate that the response was initiated on the surface of the airways. Preliminary studies using the scanning electron microscope (SEM) demonstrated that *Aspergillus* spores were taken up by the alveolar macrophage within minutes after intratracheal injection [10]. In view of the fact that macrophages exposed to a variety of organic particulates release hydrolytic enzymes that are also potent activators of complement [11], it seemed likely that the alveolar macrophage played a pivotal role in the initiation of the pathophysiological response observed in our previous experiments. The study reported here uses light and scanning electron microscopy to expand these observations further. Particular attention is paid to the rate of fungal spore phagocytosis by the alveolar macrophage and to the subsequent development of granulomatous lesions in the lungs.

MATERIALS AND METHODS

Animals

Outbred New Zealand white male 1.5–2.0 kg rabbits were obtained from Hilltop Laboratory Animals, Inc. (Scottdale, PA) and maintained on standard laboratory animal chow and water *ad libitum*. Cesarean derived (COBS) Wistar–Lewis CRL/BR 300–500 g rats were obtained from Charles River Breeding Laboratories, Inc. (Wilmington, MA), maintained in clean, respiratory pathogen-free facilities and fed sterilized animal chow.

Spores

Aspergillus terreus (ATCC 44678) was grown on polypeptone–dextrose agar for 2 weeks at 30°C in a humidified incubator. Mature elliptical conidia were harvested from the confluent growth by washing with 0.01% (v/v) Tween 80 (Fisher Scientific Co., Fairlawn, NJ) in sterile non-pyrogenic saline (Travenol Laboratories, Inc., Morton Grove, IL) and mild agitation with a sterile glass rod. The spores were washed twice with saline alone and diluted to the desired final concentration.

Intratracheal instillation

After anesthetizing the rabbits with 25–30 mg/kg pentabarbital i.v., the trachea was exposed surgically and 7.0×10^6 fungal spores were instilled in a volume of 1.0 ml as the animals inspired. Two rabbits were sacrificed by intracardiac injection of 200 mg penta-

barbital and major organs were removed at 0, 30, and 120 min after injecting the spores. Ten unexposed but similarly treated rabbits were used as controls.

Ethyl ether inhalation was used to anesthetize the rats and 5×10^7 spores in a volume of 0.25 ml were instilled intratracheally with a curved, blunt ended 20 gauge needle with direct visualization of the trachea. At each time period of 0, 15, 30 min and 3, 24, 48, 72, 96 hr and 1 week, two treated rats and one which received only a control instillation of saline were anesthetized with ethyl ether and sacrificed by exsanguination through the abdominal aorta.

Histology

The lungs of all animals were fixed rapidly *in situ* by a modification of the method of Thurston *et al.* [12] using 1% (w/v) osmium tetroxide (Fisher Scientific Co., Pittsburgh, PA) in fluorochemical liquid FC80 (Mediflor; 3M Company, St Paul, MN). All fixation procedures proceeded under a chemical fume hood. Approximately 35–40 ml (rabbits) or 8–12 ml (rats) of fixative were injected intratracheally at a moderate rate with the trachea clamped anteriorly. After injection of the fixative, the trachea was clamped posterior to the injection site and the lungs were post-fixed by flooding the chest cavity with buffered neutral formalin for 10 min. Tissue processing and removal of osmium tetroxide from the samples were reported in detail elsewhere [10]. Specimens were prepared for light microscopy and stained with hematoxylin and eosin and Gomori's methenamine silver. Sections prepared for scanning electron microscopic examinations were stained by the Willard modification of the Wilder ammoniacal silver stain [13] and observed on an ETEC Autoscan scanning electron microscope equipped with two viewing screens, a solid state backscatter electron detector and specimen current meter. Blocks of tissue were also taken from thymus, liver, spleen and kidneys and processed for routine light microscopic examination.

Antibody testing

All animals were tested for the presence of precipitating serum antibodies to *Aspergillus terreus* by counterimmunoelectrophoresis [14] against a commercially available *A. terreus* extract (Hollister-Stier Laboratories, Yeadon, PA). Positive control rabbit sera with precipitating antibodies were obtained by multiple intradermal immunization with the same fungal spores used in this study.

Macrophage uptake

Phagocytosis of spores by rat pulmonary macrophages was quantified (in silver stained sections) by light microscopy at 400×. Observations were made on two animals at each time period. Five lung sections were examined per rat and a minimum of 3500 fungal spores were counted per animal for time periods up to 3 hr. Spores associated with either the surface or cytoplasm of macrophages were considered to be in the process of phagocytosis.

Toxicity assay

Toxicity of the *Aspergillus terreus* used in these studies was assayed by the brine shrimp larvae method described by Harwig and Scott [15] with the following modifications. The tests were performed in quadruplicate in multiwell plates which contained a final volume of 1.0 ml and 20–25 larvae per well. After incubation at 25°C for 16 hr, per

cent mortality was determined by first counting dead larvae followed by counting total larvae per well after the addition of 50 μl. of formaldehyde solution (Fisher Scientific Co., Pittsburgh, PA). Whole spores as well as spore extracts were tested. Dried spores (435 mg) were extracted with dichloromethane in a Soxhlet extractor for 7 hr. The temperature in the extractor was adjusted to provide an exchange of fresh solvent every 1.5–2.0 min and a total of 3.34 mg of material was extracted.

The extractable material was used as a suspension in dimethylsulfoxide (Pierce Chemical Co., Rockford, IL) followed by serial two-fold dilutions. Dried rice (0.5 g) which was overgrown by *Fusarium oxysporum* was used as a positive toxicity control. Extraction for only 3 hr yielded 16.39 mg of material which was then used as a suspension in dimethylsulfoxide.

RESULTS

Histopathology in rabbits

Histological sections of control rabbit lungs showed moderate numbers of macrophages lying free within the alveoli and alveolar ducts. The bronchial associated lymphoid tissues (BALT) were well developed and some showed germinal centers. A mild lymphocytic cuffing of pulmonary arterioles was observed. There was no evidence of an active acute inflammatory disease process. The distributions of the spores at the three time intervals were obtained using light and scanning electron microscopy. At 0 hr spores with some contaminating fungal hyphae were observed lying on mucus patches in the trachea and major airways (Fig. 1). Spores were also observed in the small airways and in the alveoli. In the majority of the cases spores were detected in all lobes of both lungs; however, the density of spores differed between lobes in a single animal. Comparison of animals suggested an overall predilection in spores in the dorsal aspects of the lung. A moderate number of spores noted at 0 time were associated with macrophages in the alveoli and on the surface of the airways. Scanning electron microscopy of silver stained sections combined with backscattered electron imaging confirmed this finding (Fig. 2). At later time periods (30 and 120 min) the distribution of spores in the lungs remained similar though the number of macrophages associated with spores increased with time.

The rapid uptake of spores by the bronchoalveolar macrophages was an unexpected finding. Accordingly, we decided to repeat the experiments in rats to determine the following: (1) whether the phenomena are confined to a single species; (2) the rate of uptake of spores by macrophages; and (3) the eventual fate of the spores and the possible development of pulmonary lesions.

Histopathology and quantification of spore uptake in rats

The lungs of the control rats showed very few macrophages within the alveoli, no lymphocytic cuffing of pulmonary arterioles and small BALT without germinal centers. The distribution of spores in the lungs of exposed rats was fairly uniform and did not show the predilection for the dorsal areas seen in the rabbits. At 0 time spores were observed on the surface of the airways and on the surfaces of the alveolar ducts and alveoli. As in the rabbits, a few spores were seen in association with bronchoalveolar macrophages at this time interval. Thereafter, the number of macrophages seen in the alveoli and on the surface of the airways by light and scanning electron microscopy had increased and the number of free spores had decreased. At times after 3 hr very few free

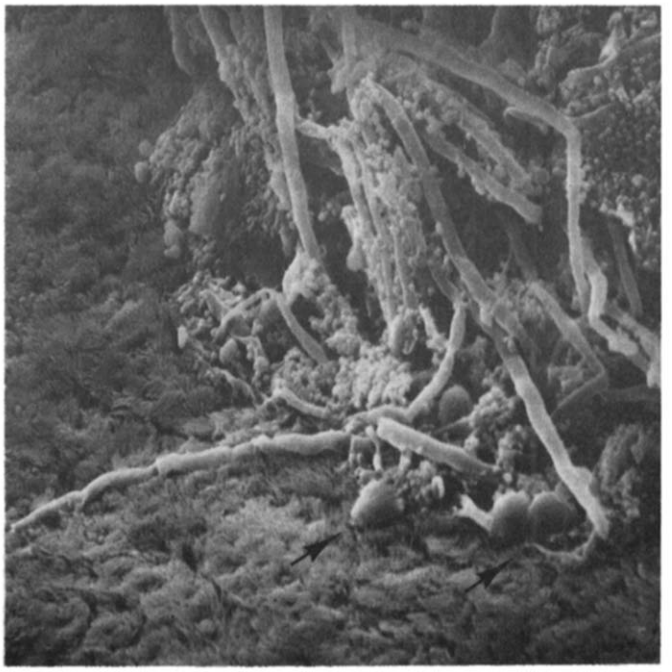
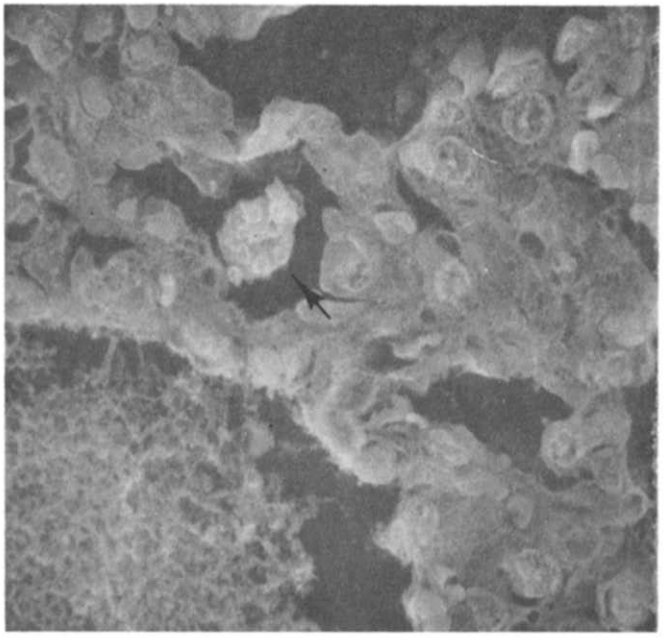
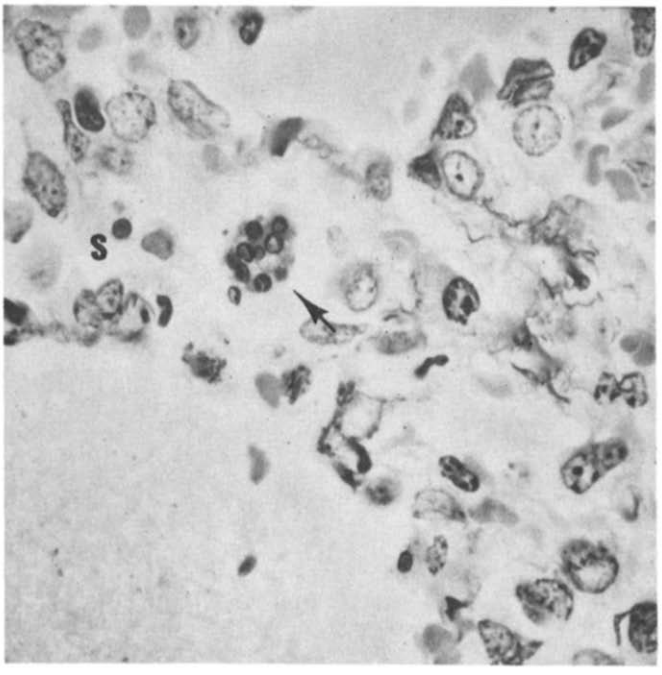


Fig. 1. Scanning electron micrograph of rabbit trachea immediately after exposure. Clumps of spores with contaminating hyphae are seen on the ciliated surface epithelium. Several macrophages (arrows) are infiltrating the clump.



(a)



(b)

Fig. 2. Seven μm section of rabbit peripheral lung viewed immediately after exposure, stained with modified Wilder's ammoniacal silver stain [13]. (a) Secondary electron image showing macrophage within alveolus (arrow). (b) Backscattered electron image showing black spores within cytoplasm of the macrophage (arrow). A spore is also seen within the interstitium (S) $\times$ 1000.

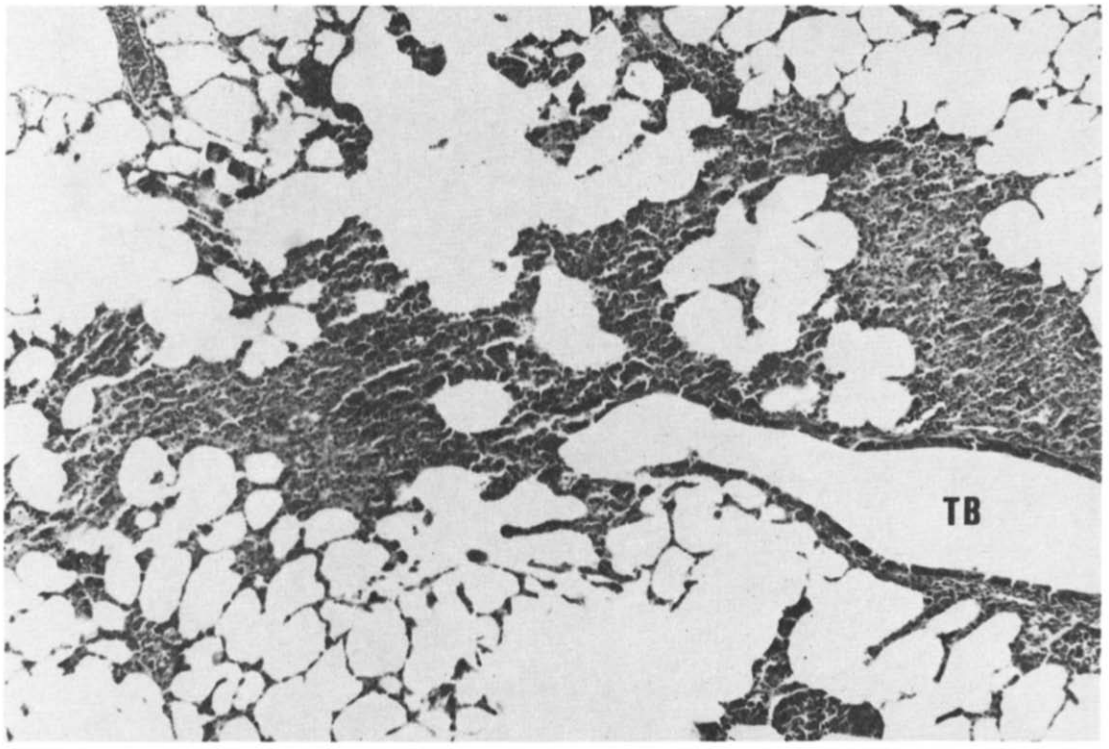


Fig. 3. Rat lung viewed 48 hr following intratracheal injection of *Aspergillus* spores. The alveoli distal to a terminal bronchiole (TB) are filled with masses of spore containing macrophages and polymorphonuclear leukocytes. Hematoxylin and eosin $\times 24$.

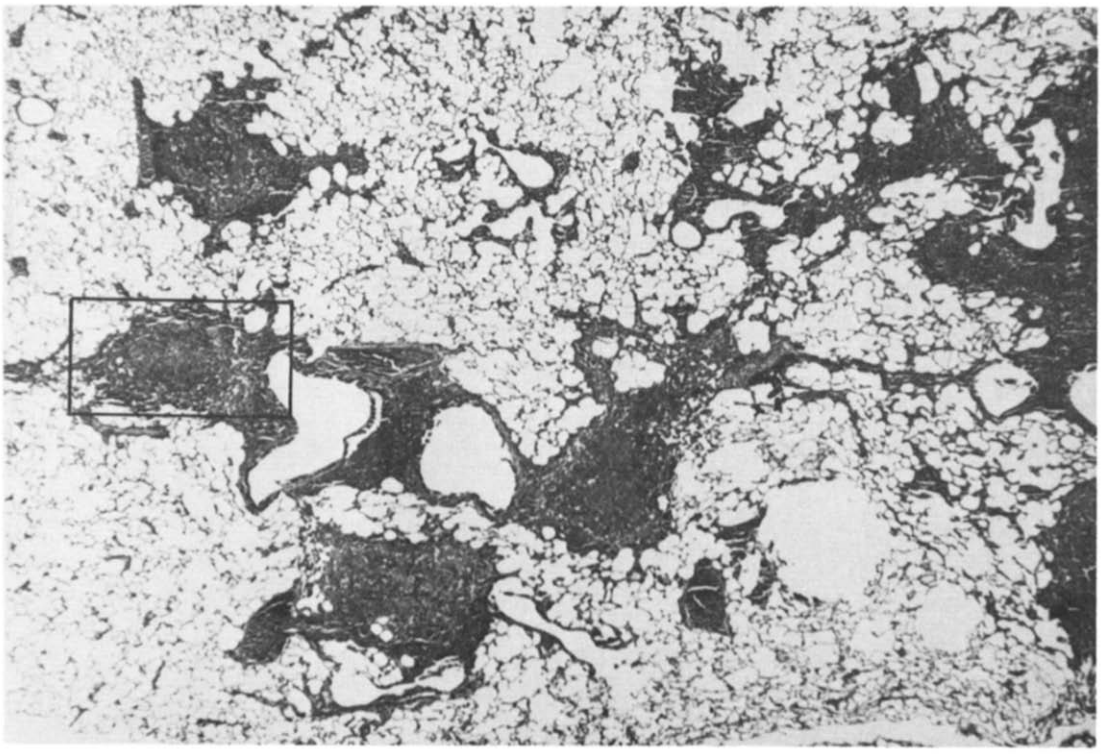


Fig. 4. Low power view of rat lung one week following intratracheal injection of *Aspergillus* spores. Numerous granulomata are seen distributed throughout the lung parenchyma. Hematoxylin and eosin × 12.

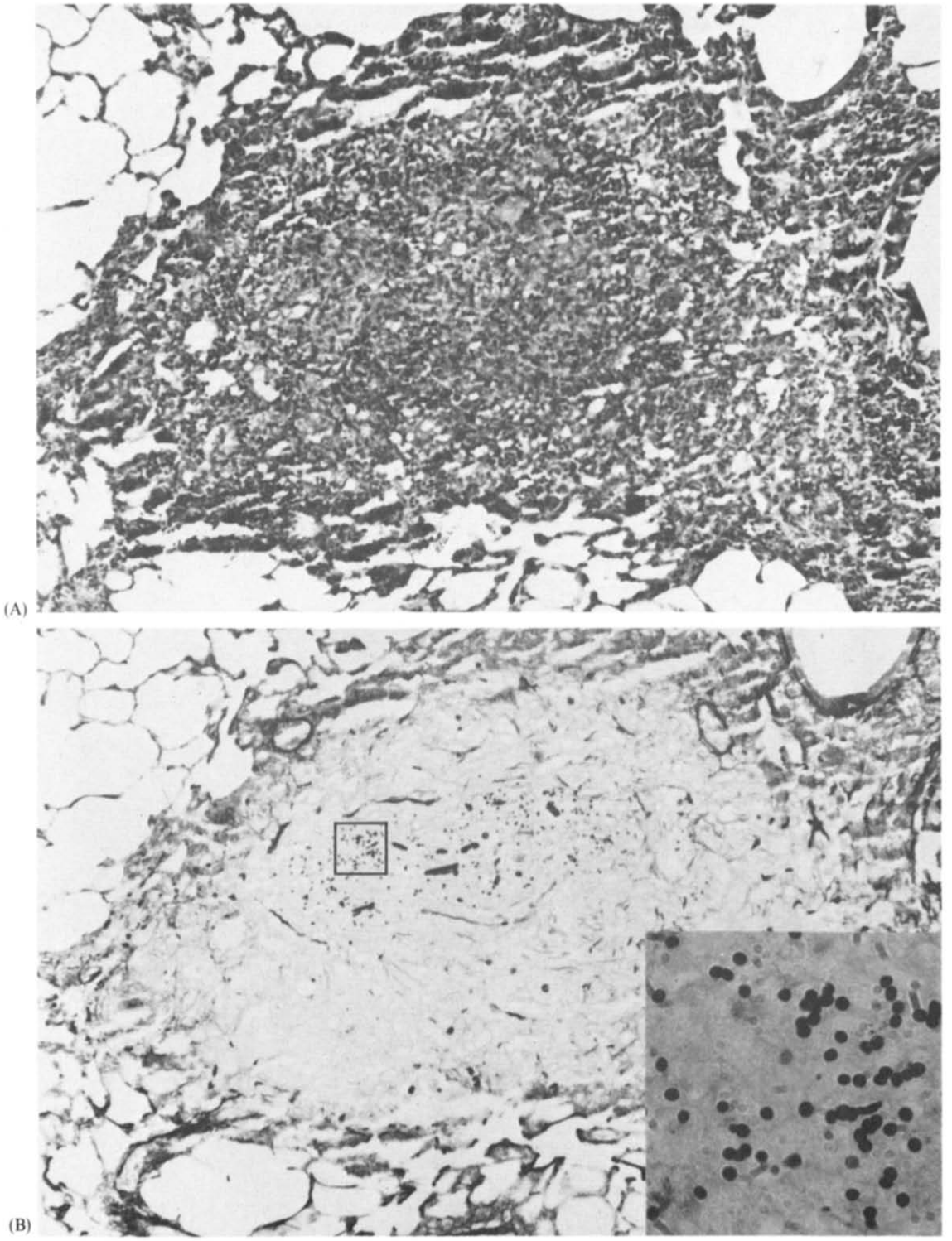


Fig. 5. Higher magnification of granuloma outlined in Fig. 4. (A) The granuloma is composed of macrophages, giant cells and lymphocytes. (B) Silver stain shows fungal spores and fragmented hyphae at center of lesion. Inset: high power view of spores. Hematoxylin and eosin and Gomori's methenamine silver stains, × 75.

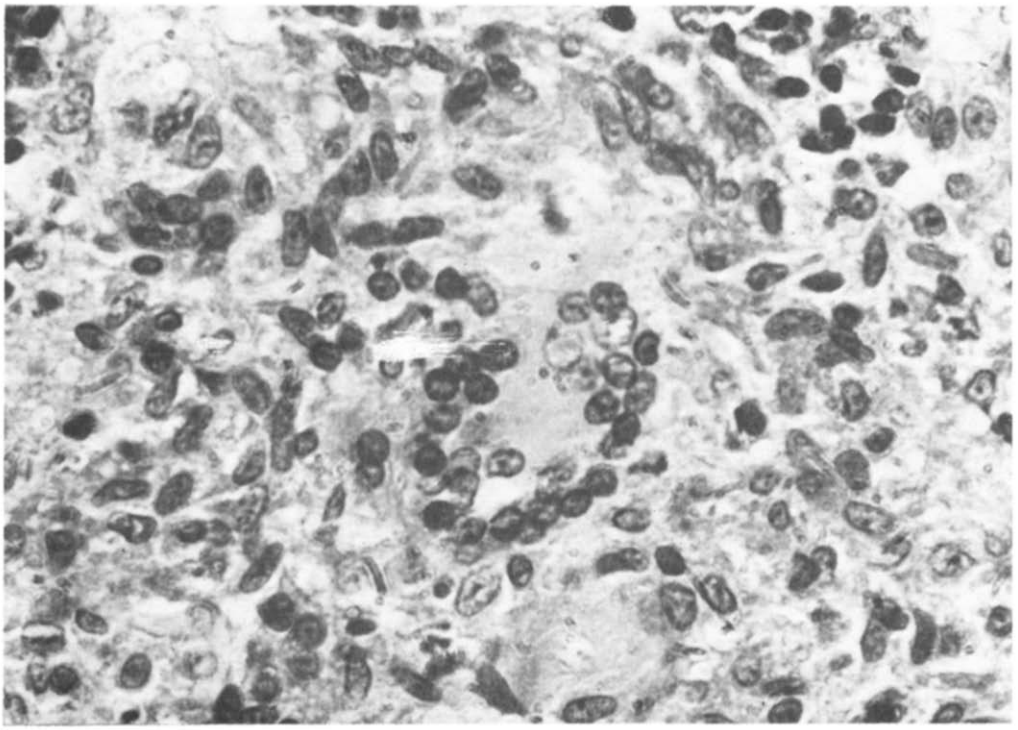


Fig. 6. High power view of center of a granuloma showing multinucleate (Langhan's type) giant cells. Hematoxylin and eosin ×480.

Table 1. Rate of phagocytosis of *Aspergillus terreus* spores by rat alveolar macrophages

Time	% Associated with macrophages*	Total no. of spores observed
0	42	3518
15 min	69	4667
30 min	78	4241
3 hr	84	4171
24 hr	98	816†

* Observations on 2 animals and 5 sections per lung.

† Low number assumed a function of clearance.

spores were discernable. An estimation of the rate of phagocytosis of spores is shown in Table 1.

Macrophages were the predominant reactive cell and polymorphonuclear cells were less frequently observed at the early time intervals. The majority of the spores were seen within macrophages on the surfaces of airspaces. A small number of spores were identified within the alveolar interstitium at 3 hr and spores were detected in the tracheobronchial lymph nodes at 24 hr and later periods. Spores were not observed penetrating the alveolar or bronchial epithelium, nor were spores observed in the specialized epithelial cells (M cells) overlying the BALT. Between 3 and 48 hr there appeared to be a migration of macrophages from the peripheral alveoli to the alveoli adjacent to the respiratory bronchioles. In the latter location, large numbers of macrophages containing spores and lesser number of polymorphonuclear cells were observed completely filling the alveoli (Fig. 3). Macrophages containing spores were also observed on the ciliated epithelial surfaces of the bronchioles.

At time intervals between 48 hr and 1 week the majority of these macrophage aggregates dissolved. A proportion, however, persisted and became rounder in outline (Fig. 4). At higher magnification the lesions appeared granulomatous and were composed of macrophages, Langhan's type giant cells and occasional polymorphonuclear cells and lymphocytes (Figs 5 and 6). Central necrosis was seen in some lesions. The relative proportion of lymphocytes increased over the 7-day period. Initially the spores were randomly distributed throughout the lesions, but in later lesions the spores were predominantly centrally placed (Fig. 5B). Control animals showed none of the above features. Sections from thymus, liver, spleen and kidney of both test and control animals showed no abnormalities.

Antibody results

All animals used in this study were negative for serum percipitating antibodies against *A. terreus* extract. Positive control sera yielded 5–6 lines of precipitation after counterimmunoelectrophoresis.

Toxicity of Aspergillus terreus

Results of the brine shrimp larvae toxicity assay are shown in Tables 2 and 3. The

Table 2. Toxicity of *Aspergillus terreus* spores as measured by the brine shrimp assay

Spores (number/ml)	Mortality (16 hr) Plate 1	Plate 2
None	1.2*	2.1
1.3×10^5	5.3	1.4
1.3×10^6	2.5	2.7
1.3×10^7	1.9	1.6
1.3×10^8	0.0	0.0
1.3×10^9	—†	†

* Replicate wells/plate.
† Could not count larvae because spores created an opaque layer in each well.

spores alone (Table 2) showed only little toxicity above the baseline level and could be considered negative in this assay. Likewise, the dichloromethane extraction of the spores produced little excess toxicity. Table 3 shows significant toxicity in the positive control extract of *Fusarium oxysporum* and none in the *A. terreus* tests.

DISCUSSION

This study expanded our previous work [10] and provided details of the medium-term fate of inhaled *Aspergillus* spores. The rabbits, although unimmunized and without overt disease, showed relatively developed BALT, some with germinal centers, mild lymphocytic cuffing of pulmonary arteries, as well as a moderate number of macrophages on the alveolar surfaces. We therefore chose to use a cleaner animal, cesarean-derived specific pathogen free rats, in order to permit observations of any subtle tissue changes which might occur after exposure to *Aspergillus* spores. Although the histopathology in rabbits and rats was similar, a difference in the distribution of the fungal spores was noted between the two species, with dorsal spore concentration in the rabbit. This deposition,

Table 3. Toxicity of *Aspergillus terreus* spore extract as measured by the brine shrimp assay

Extract dilution	Mortality (16 hr) (%) *A. terreus*	*F. oxysporum*
1:1	0.0*	74.4*
1:2	1.5	71.2
1:4	3.8	72.6
1:8	0.0	88.9
1:16	1.5	55.4
1:32	1.9	8.7
0	2.1†	2.1†

* Four replicate wells/dilution. *F. oxysporum* used as positive control.
† Nineteen replicate wells.

however, may more accurately reflect differences in intratracheal instillation procedures than in species-related effects.

Quantification of our earlier observation that spores are taken up rapidly by the pulmonary macrophage showed that 42% of the observed spores were associated with macrophages immediately after instillation. Within 30 min post-exposure, 78% of the spores were macrophage-associated while nearly all spores were associated with the macrophages at 24 hr. These results indicate that the alveolar macrophage, combined with the mucocilliary escalator, provides an efficient first line of defense against inhaled organic material. At the same time, as suggested by Brain [16], the pulmonary macrophage may transport and present antigen to the pulmonary lymphoid tissue. Our study showed, in the absence of obvious direct penetration of the lung architecture by the spores, a small number of spores within the alveolar interstitium at 3 hr. By 24 hr spores were also found in the tracheobronchial lymph nodes. This clearance route provides for immune reactivity, and the development of granulomas at the 7 day interval raises the possibility that these lesions were a consequence of hypersensitivity.

These granulomas were one of the more significant observations of this study. Whether these lesions formed as a result of a hypersensitivity reaction, or as a direct result of the pathogenicity of the fungal spores cannot be determined from these studies. The absence of an antibody response to the spores precludes the first possibility and furthermore, there was no evidence of an active infection. The brine shrimp assay was negative for toxic metabolites both with whole spores and spore extract. Therefore, direct toxicity of the inhaled spores probably would not cause the tissue reactions we observed. It is of course possible that agents which are not toxic in the brine shrimp larvae assay could be toxic for animal tissues. The brine shrimp larvae assay is a screening test for toxicity and not necessarily indicative of animal cell–spore reactions. The granulomas formed at the points of maximum concentration of the spores in the region of the respiratory bronchioles. An overload of the clearance mechanisms at this point may be associated with release of lysosomal enzymes from injured macrophages and direct complement activation which could have led to the central necrosis observed in the macrophage aggregates. In this regard, the pulmonary macrophage, while for the most part efficiently protecting the lung from inhaled antigen, may under certain circumstances also initiate pulmonary injury.

The model system which we used in this study together with our previous studies have shown that *Aspergillus terreus* is able to initiate a number of different pathophysiologic responses in animal lungs [7, 8]. Heavy respiratory exposures to *Aspergillus* species can be found in many occupations, and several clinically distinct disease entities related to *Aspergillus* spore inhalation have been described [2–6]. In one of these diseases, hypersensitivity pneumonitis, the formation of pulmonary granulomas, is considered pathognomonic [17]. Thus, the disease model described here may be of future value for characterizing further the pathology of this form of occupational lung disease.

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