STUDIES ON THE PATHOGENESIS OF EXPERIMENTAL PULMONARY ASPERGILLOSIS

SHELDON M. EPSTEIN, M.D., ETHEL VERNEY, M.S.,
THOMAS D. MIALE, A.B.,* AND HERSCHEL SIDRANSKY, M.D.

From the Department of Pathology, University of Pittsburgh School of Medicine Pittsburgh, Pa.

Many reviews [1-10] in recent years have reported a progressive increase in the prevalence of secondary infections due to opportunistic fungi such as *Aspergillus, Mucor,* and *Candida.* Since these secondary mycotic infections occur often in acutely or chronically ill patients, and in the course of a variety of disease conditions, for which potent therapeutic agents such as corticoids, broad-spectrum antibiotics, irradiation, and cytotoxic drugs have been used, numerous factors have been implicated to be of importance in the pathogenesis of secondary mycotic infections.

Experimental studies [11-21] have been undertaken in an attempt to gain some knowledge of the pathogenesis of these widely prevalent opportunistic fungal infections. Many of these studies have been primarily oriented toward reproducing secondary mycotic disease in the laboratory animal by utilizing agents or conditions that are frequently encountered in human cases. Prior experimental studies from this laboratory [17,20,21] have demonstrated that mice exposed to the inhalation of aerosols of dry, viable spores of *Aspergillus flavus* become highly susceptible to fatal pulmonary aspergillosis when subjected to conditions frequently associated with human cases of lethal aspergillosis or treated with agents used in the clinical management of patients who develop the disease. Therapeutic agents such as corticosteroids, cytotoxic drugs, and roentgen radiation, as well as various experimental regimens such as feeding protein deficient diets, the presence of transplantable tumors, or alloxan diabetes mellitus, rendered normal mice highly susceptible to hyphal bronchopneumonia following the inhalation of *A. flavus* spores. On the other hand, normal control mice exposed to spores in a similar manner

Supported by Research Grants AI-05519 from the National Institute of Allergy and Infectious Diseases, and GM-10269 from the National Institute of General Medical Sciences, U. S. Public Health Service.

Presented in part at the Annual Meeting of the Federation of American Societies for Experimental Biology, Atlantic City, Apr. 9–14, 1965, and at the Annual Meeting of The American Association of Pathologists and Bacteriologists, Washington, Mar. 10–12, 1967.

Accepted for publication July 3, 1967.

* Medical student research trainee supported by Training Grant 5 T5 GM47 from the National Institute of General Medical Sciences, U.S. Public Health Service.

were highly resistant to pulmonary infection. Thus, under selected experimental conditions the mouse becomes a suitable model for the study of pulmonary aspergillosis resembling that found in man.

The present study was concerned with the early stages in the pathogenesis of experimental pulmonary aspergillosis in cortisone-treated mice. In an attempt to obtain further insight into some of the mechanisms involved at the cellular level, selected aspects of morphologic and functional alterations occurring within the first 12 hr. after exposure to spores were studied and form the subject of this communication.

Materials and Methods

General Methods

White female mice (CF1 strain) from Carworth Farms, weighing on the average 20 gm., were used in most of the experiments. The cortisone-treated mice received 5 mg. cortisone acetate subcutaneously 2 days prior to spore exposure. In a few experiments the mice weighed 30 gm. and received 7.5 mg. instead of 5 mg. cortisone acetate. In all experiments, untreated control mice were exposed to spores simultaneously with the cortisone-treated mice.

The exposure chamber was similar to that described by Piggott and Emmons[22] and had been used previously in our laboratory.[20,21] Twenty mice were exposed simultaneously in the chamber. The spores were the same strain of *A. flavus* as used in earlier studies[17,20,21] and had been cultured originally from a case of human aspergillosis.[23] Spores were collected by vacuum suction from dried cultures of *A. flavus* and were sprayed into the chamber with a powder atomizer. The mice inhaled spores for 5 min. In an attempt to keep exposure uniform, 3 sprays from the powder atomizer were administered into the chamber at zero time, another single spray after 2 min., and a fifth spray was given after 4 min. To determine the approximate number of viable spores retained within the lungs during exposure, 1 or 2 animals were killed immediately upon removal from the chamber and the left lung of each was removed aseptically. Each lung was homogenized in sterile saline, and suitable dilutions were plated on Sabouraud's dextrose agar. As in earlier experiments,[20,21] the mice were found to retain 2×10^5 to 3×10^5 viable spores per left lung.

Peribronchiolar Acute Inflammatory Cell Response

In experiments designed to study the acute inflammatory cell reaction in the lungs following exposure to spores, cortisone-treated and control mice were exposed to spores and killed either immediately or after 6 or 9 hr. The degree of peribronchiolar acute inflammatory cell reaction was evaluated by examining lung sections stained with hematoxylin and eosin. Quantitative measurement was undertaken by counting the number of leukocytes that were present adjacent to each of at least 10 terminal bronchioles of each mouse. This value was then divided by the number of epithelial cells covering the bronchioles. The lungs of each animal were thus scored by this value (average peribronchiolar inflammatory cell index).

Cytologic Study of Alveolar Macrophages

In experiments in which the cytology of tracheobronchiolar washings was studied, cortisone-treated and control mice were killed immediately, at 6, 9, 12, or 16 hrs. following exposure. The technique for obtaining tracheobronchiolar washings from the lungs of mice was that described by Pinkett, Cowdrey, and Nowell.[24] Animals were killed by cutting the abdominal aorta. Following rapid exsanguination, the trachea

was isolated and a needle (23- or 26-gauge) was inserted and then tied in place with a ligature. Lavage was performed by repeated washings (3–5 times) with 0.5–1.0 ml. of isotonic saline or 0.25 M sucrose. The pooled washings were centrifuged and the cells (90% alveolar macrophages) were then studied. The morphologic appearance of the alveolar macrophages in the tracheobronchiolar washings of all groups of animals was identical to granular pneumocytes (Type II alveolar cells).

Acid Phosphatase Studies

In some experiments alveolar macrophages were assayed for acid phosphatase. In these studies aspirated macrophages were obtained from cortisone-treated and control mice 8 hr. after exposure to spores. The lungs of each animal were lavaged with 1.0 ml. of 0.25 M sucrose containing 0.002 M EDTA (disodium ethylenediaminetetraacetate). Homogenization and centrifugation were performed using the technique described by Cohn and Wiener [25] with slight modifications. In preliminary experiments the acid phosphatase activity in the nuclear fraction, which contained some unbroken cells (10–15% of total macrophages), was found to be only 10–15% of the total activity of the macrophages from both control and experimental animals. Therefore, in subsequent experiments this fraction was not assayed. Aliquots of the pooled washings from the lungs were taken for cytologic study and for cell counts using a white blood cell counting chamber. The remainder was homogenized with a Teflon pestle for three 90-sec. periods, separated by equal periods on ice. Following homogenization an aliquot was removed for assay of the acid phosphatase activity of the whole homogenate. The rest of the homogenate was centrifuged at 15,000 *g* for 12 min. and the supernatant fraction was decanted. The precipitate (the "particulate fraction") obtained after centrifugation at 15,000 *g* for 12 min. was resuspended in 0.25 M sucrose containing 0.002 M EDTA. The whole homogenate, the supernatant fraction, and the particulate fraction were each frozen and thawed 10 times. Following this, all three were assayed for acid phosphatase activity. Each was incubated with 0.05 M sodium acetate buffer, pH 5.0, with 0.05 M sodium beta glycerophosphate as substrate for 75 min. at 37.5° C.[26] The reactions were stopped with 2.0 ml. of 5% trichloroacetic acid, and following centrifugation the supernatants were assayed for inorganic phosphorus.[27] When necessary, lipid was extracted with ether to yield a clear supernatant. In preliminary experiments the total activity of acid phosphatase of alveolar macrophages from unexposed cortisone-treated and control mice was found to be the same when adjusted to a fixed number of cells (1.2–2.5 μg. of phosphorus liberated per hour per million cells for each group). Also the supernatant fraction of alveolar macrophages of unexposed control and experimental mice had a similar percentage of enzyme activity (5–20%).

Studies of Spores and Macrophages in Vitro

Some experiments were designed to study in vitro the ability of alveolar macrophages to kill spores. In each in-vitro experiment an equal number of macrophages (6.4×10^4 to 32×10^4) from control and cortisone-treated mice was incubated with an identical spore inoculum (2.9×10^4 to 6.2×10^4) in 1.0 ml. of 0.1 M phosphate buffer (pH 7.4) containing 1 mg. glucose for 75 min. at 37.5° C. Suitable dilutions were then taken and plated on Sabouraud's dextrose agar to determine the number of viable spores remaining after the incubation.

Protein-Bound Trypan Blue Studies

In experiments designed to study the degree of extravascular protein extravasation within the lungs following exposure to spores, trypan blue (Allied Chemical Lot 673-P), 0.3 ml. of a 1% solution in saline was injected intraperitoneally into control and cortisone-treated mice 8 hr. following exposure to spores and 45 min. later the animals were killed by exsanguination. Some animals received dye but were not ex-

posed to spores. The extraction and estimation of trypan blue in the lungs were performed according to the technique of Judah and Willoughby.[28] Results were expressed as the percentage increase in optical density per gram of lung of the exposed mice as compared to the optical density per gram of lung of control unexposed mice.

Studies of the Effect of Leukocytosis on Aspergillosis

In experiments designed to study the influence of altering the circulating leukocyte levels on the response of control and cortisone-treated mice to spore exposure, pertussis vaccine was administered to increase the circulating leukocytes. In these experiments mice were given 0.2 ml. of Lederle Phase One pertussis vaccine (containing 8 U./ml.) in the tail vein. Three days after receiving the vaccine, some mice were also given 5 mg. cortisone acetate subcutaneously. All of the mice that received pertussis vaccine were exposed to spores 5 days after receiving the vaccine. In addition, 2 control groups of mice were exposed: one that received cortisone acetate 2 days prior to spore exposure but had received no pertussis vaccine, and another group that received neither pertussis vaccine nor cortisone. The mice were necropsied immediately after death or after they were killed 4 weeks following spore exposure.

Histologic and Cytologic Methods

For routine histologic study the lungs were fixed in 10% formalin, and paraffin sections were stained with hematoxylin and eosin and with the Gridley stain.[29] Alveolar macrophages obtained from the tracheobronchial washings were stained with hematoxylin and eosin and with the Jenner-Giemsa stain.[30] Leukocyte counts were determined on tail vein blood.[21]

RESULTS

Peribronchiolar Acute Inflammatory Cell Response after Exposure to Spores

The results, expressed as average peribronchiolar inflammatory cell index of the lungs, of control and cortisone-treated mice killed 0, 6, and 9 hr. after exposure are summarized in Table I. When animals were killed immediately upon removal from the exposure chamber, the lungs of the control and cortisone-treated mice appeared the same histologically. The lungs of both groups of mice had some slight peribronchiolar acute inflammatory cell infiltrate. The inflammatory cell infiltrate in the

TABLE I

PERIBRONCHIOLAR INFLAMMATORY RESPONSE IN THE LUNG OF MICE TO *A. flavus* SPORES

	Peribronchiolar inflammatory reaction at indicated times after spore exposure *		
Group	*0 hr.*	*6 hr.*	*9 hr.*
Control	6.8±1.4 (8) †	9.4±1.1 (11)	12.9±0.6 (18)
Cortisone-treated	5.4±0.6 (8)	7.5±0.7 (12)	8.6±0.7 ‡ (18)

* Score of peribronchiolar inflammatory reaction: No. of adjacent WBCs/No. of bronchiolar epithelial cells × 10; mean ± S.E.

† No. of mice (4 experiments) in parentheses.

‡ Differs from nine hour control by $p < 0.01$.

lungs of control and cortisone-treated mice killed 6 hr. after spore exposure also revealed no significant differences. However, at this time interval, in rare cases germ tube elongation indicating early spore germination, best seen on the Giemsa-stained tissue slides, was present within the lungs of the cortisone-treated but not of control animals. When mice were killed 9 hr. after spore exposure, a significant decrease ($p < 0.01$) in the peribronchiolar leucocytic infiltrate (Table I) was seen in the lungs from cortisone-treated animals (Fig. 1) when compared to the lungs of control mice (Fig. 2). In addition, Giemsa-stained lung sections of cortisone-treated mice revealed evidence of frequent spore germination (Fig. 3) while the lungs of control mice contained spores but no evidence of germination.

Studies on Alveolar Macrophages

Morphologic Studies. Tracheobronchial washings of control or cortisone-treated mice obtained 15 min. after spore exposure contained alveolar macrophages with intracellular spores. The washings obtained from cortisone-treated mice 6 hr. after exposure revealed extracellular spore germination (Fig. 4) as well as early germination of spores within alveolar macrophages (Fig. 5). In contrast, washings obtained from control mice revealed that many spores had been phagocytosed by alveolar macrophages, but germination either intracellularly or extracellularly was relatively infrequent. Sixteen hours after exposure the washings obtained from cortisone-treated mice contained many large aggregates of spores and hyphae (Fig. 6) which sometimes obstructed the aspirating needle. Alveolar macrophages, some of which displayed nuclear pyknosis, contained only a minority of the aspirated spores. Washings from control mice at this time showed a few extracellular spores, but many more spores were within macrophages, and germination was rarely seen. Intracellular spores in control animal aspirates were now of varying shape and size with many being decreased in size. There was a frequent decreased affinity of the periphery of these small intracellular spores for basophilic stains (Fig. 7).

Enzyme Studies. Acid phosphatase activity was assayed in the particulate and supernatant fractions of alveolar macrophages from control and cortisone-treated mice. Eight hours after exposure to spores, the alveolar macrophages were obtained by tracheal lavage. Washings obtained from both groups of mice contained a uniform cell population (approximately 90% macrophages). A similar percentage of alveolar macrophages containing spores was present in washings obtained from both groups.

Alveolar macrophages obtained from control mice 8 hr. following in-

vivo exposure had 67% of the acid phosphatase activity in the supernatant fraction with 37% of the acid phosphatase activity in the particulate fraction. In contrast, the acid phosphatase activity of alveolar macrophages obtained from cortisone-treated mice exposed to spores was present mainly (78%) in the particulate fraction with only 23% of the enzyme activity in the supernatant fraction. Recovery of enzyme activity, obtained by adding the activities found in the particulate and supernatant fractions, was complete in both control (104%) and experimental (101%) animals when the sum was compared to the activity in an aliquot of the unfractionated homogenate. The data from 8 experiments are summarized in Table II.

Since unbroken spores were found to settle in the particulate fraction, some experiments were performed in which spores without cells were homogenized and incubated under our experimental conditions. No detectable acid phosphatase activity was found even when the spore concentration was increased many fold over that found within mouse tracheobronchiolar washings following in-vivo exposure. Furthermore, the addition of spores to frozen and thawed aliquots of alveolar macrophages (whole homogenate, particulate fraction, or supernatant fraction) did not alter the acid phosphatase activity when compared to the activity of the same fractions without addition of spores.

An average of 0.6–0.7 ml. of tracheobronchiolar washings was obtained from each mouse. Macrophage cell counts on the washings revealed a concentration of 0.28×10^6 to 1.1×10^6 cells per milliliter. Therefore, it was necessary to kill 16–36 mice in order to obtain 28×10^6 alveolar macrophages which were usually used to assay acid phosphatase activity. The enzyme activity was shown to be linear over the range of alveolar macrophage concentration used experimentally.

Studies on the Viability of Spores Incubated in Vitro with Alveolar Macrophages. The effect on viability of spores after in-vitro incuba-

TABLE II

ACID PHOSPHATASE ACTIVITY IN PARTICULATE AND SUPERNATANT FRACTIONS OF MICE ALVEOLAR MACROPHAGES 8 HR. AFTER IN-VIVO EXPOSURE TO VIABLE *A. flavus* SPORES

Group	*Acid phosphatase activity in alveolar macrophages* *		
	Particulate fraction (A) % †	*Supernate fraction (B)* % †	*Recovery* (Σ *A+B)* % †
Control	37.0±4.2 ‡	66.9±11.1	103.9±8.8
Cortisone-treated	78.4±6.2 §	22.7±6.1 §	101.1±4.4

* Data from 8 experiments.
‡ Mean ± S.E.
§ $P<0.01$.
† Percentage of activity as compared to whole homogenate.

TABLE III

VIABILITY OF *A. flavus* SPORES AFTER IN-VITRO SPORE INCUBATION WITH ALVEOLAR MACROPHAGES

Exper. No.	*Viable* A. flavus *spores after 75 min. in-vitro incubation with macrophages obtained from:*	
	Control mice × 10^5	*Cortisone-treated mice* × 10^5
1	39 *	56 (+42%) †
2	52	66 (+28%)
3	28	40 (+42%)

* Each value is the mean of three incubations.

† Percentage in parentheses refers to viable spores as contrasted to those incubated with control alveolar macrophages.

tion of an equal number of alveolar macrophages from control and cortisone-treated animals with an equal number of spores was tested. Cytologic study revealed that a similar number of spores had been phagocytized by macrophages of the 2 groups of animals. Incubations were carried out in triplicate in each experiment using control and experimental macrophages. The results expressed as viable spores remaining after 75 min. incubation are summarized in Table III. In 3 separate experiments a greater number of viable spores remained after incubation with alveolar macrophages from cortisone-treated mice than after incubation with macrophages of control mice.

Studies on Dye (Trypan Blue)-Bound Protein Extravasation in the Lungs Following Spore Exposure

Protein extravasation into the lungs following exposure to spores was assayed by means of the dye, trypan blue. The dye concentration in the lungs was similar in cortisone-treated and control mice not exposed to spores (94.5% ± 12.1%; 100% ± 5.8%, respectively, based on 14 determinations for each group). However, control mice 8 hr. following spore inhalation had a high dye concentration (1214%) (Table IV). In contrast, the lungs of cortisone-treated mice similarly treated with dye

TABLE IV

TRYPAN BLUE IN MOUSE LUNG 8.75 HR. AFTER IN-VIVO EXPOSURE TO *A. flavus* SPORES

Group	*No. of determinations (9 exper.)*	*Lung trypan blue concentration * (Mean ± S.E.)*
Control	21	1214 ± 316
Cortisone-treated	20	150 ± 26 †

* Expressed as percentage of increase in optical density per gram of lung as compared to control mice given simultaneous trypan blue injections but not exposed to spores. Non-exposed cortisone-treated mice had a concentration of 94.5 ± 12.1% trypan blue in the lungs.

† $p < 0.01$.

had only 150% more dye than unexposed mice given dye injections. The extracellular distribution of the trypan blue in the lungs was confirmed by examining unstained tissue sections.

Influence of the Number of Circulating Leukocytes on Lethal Pulmonary Aspergillosis

Since cortisone treatment induces a decrease in circulating leukocytes,[31] it became important to determine whether this leukopenia was of importance in the development of lethal pulmonary aspergillosis. Control and cortisone-treated mice were treated with pertussis vaccine, which induces a marked leukocytosis,[32] and then exposed to spores.

Control or cortisone-treated mice that received pertussis vaccine had an elevated blood white cell concentration on the day of spore exposure when compared to control or experimental mice that received no vaccine (Table V). In addition, both groups of mice that received pertussis vaccine had a similar elevation of white blood cell concentration (42,000/cu.m.) 2 days after spore exposure. Mortality associated with hyphal bronchopneumonia in mice receiving cortisone but not pertussis vaccine prior to exposure was 83%. A similar incidence (82%) was found in these cortisone-treated mice that received pertussis vaccine and had an associated leukocytosis. The hyphal bronchopneumonia in both groups receiving cortisone was quite extensive and was similar to that previously described.[17,20,21] In contrast, the hyphal bronchopneumonia in the mice receiving pertussis vaccine, but no corticosteroid (16%) was histologically quite minimal. In the latter mice little vascular or parenchymal penetration by hyphae was observed. Gross and microscopic evidence of

TABLE V

INFLUENCE OF PERTUSSIS VACCINE-INDUCED LEUKOCYTOSIS ON THE INDUCTION OF FATAL HYPHAL BRONCHOPNEUMONIA IN CORTISONE-TREATED MICE EXPOSED TO *A. flavus* SPORES

Treatment	*No. of mice*	*WBC at time of spore Exposure (%)**	*Mortality at end of 4 wk.*		*Hyphal bronchopneumonia in dying mice*	
			No.	*%*	*No.*	*%*
Pertussis vaccine †	56	406	21	37.5	9	16.1
Pertussis vaccine † and cortisone acetate ‡	67	315	60	89.6	55	82.1
Cortisone acetate ‡	18	43	17	94.4	15	83.3
None	23	100	1	4.3	0	0

* As % of nontreated control mice.

† Intravenous administration of 0.2 ml. of Lederle Phase 1 pertussis vaccine performed 5 days prior to spore exposure.

‡ Subcutaneous injection of 5 mg. cortisone acetate given 2 days prior to spore exposure.

pulmonary artery embolization was encountered in these animals, and this probably arose from the tail veins where they had received the pertussis vaccine.

Discussion

This study has demonstrated that within several hours after exposure to spores of *A. flavus*, significant changes develop in the lungs of cortisone-treated mice which may be of major importance in the subsequent development of lethal hyphal bronchopneumonia. The early significant pulmonary morphological findings (summarized in Table VI) are rapid spore germination and a decreased peribronchiolar inflammatory cell response in the experimental mice. In addition, vascular permeability in the lungs of cortisone-treated mice is significantly less than that in the lungs of control mice. Our data can best be integrated by considering the hypothesis that alterations of lysosomal membranes are of etiologic significance in the pathogenesis of the early changes in experimental pul-

Table VI

OBSERVATIONS ON PULMONARY HISTOLOGY AND ON CYTOLOGIC EXAMINATION OF TRACHEOBRONCHIAL WASHINGS OF CONTROL AND CORTISONE-TREATED MICE AFTER IN-VIVO EXPOSURE TO *A. flavus* SPORES

Time after exposure (hr.)	*Group*	*Pulmonary histologic findings*		*Cytological findings of alveolar macrophages and fungus*
		Peribronchiolar inflammation	*Fungus*	
<1/2	Control	o to +	Spores	Intracellular (+) spores
	Cortisone-treated	o to +	Spores	Intracellular (+) spores
6	Control	+ to ++	Spores	Intracellular (++) spores
	Cortisone-treated	+ to ++	Spores and germ tubes	Intracellular (++) spores, some intra- and extraecllular germ tubes
9	Control	+++	Spores	Intracellular (+++) spores, some spores encased by vacuoles
	Cortisone-treated	++	Spores and germ tubes	Intracellular (+++) spores, some intra- and extracellular germ tubes
16	Control			Intracellular (++++) spores, some cells containing up to 7 spores
	Cortisone-treated			Intracellular (++) spores; many extracellular (++) spores; germ tubes and hyphae; some macrophage nuclear pyknosis
24	Control	+++	Spores; rare hyphae	
	Cortisone-treated	++++	Many hyphae, focal necrosis, parenchymal invasion	

monary aspergillosis. Major support for this hypothesis is derived from the data of the experiments with alveolar macrophages. The decreased release of lysosomal factors, as assayed by acid phosphatase activity in macrophages obtained from steroid-treated animals was considered to be of significance. In addition, the alveolar macrophages of these animals showed a diminished ability to kill or prevent germination of phagocytosed spores both in vivo and in vitro. The present findings offer experimental evidence in support of the earlier working hypophesis of others[33–35] that cortisone may act to stabilize lysosomal membranes and that this action may be of importance in the pathogenesis of an infectious disease. The present results actually appear to be the first demonstration that alterations in lysosomes may be directly implicated in the pathogenesis of an experimental disease.

The decreased peribronchiolar inflammatory cell infiltrate observed in the experimental animals 9 hr. after exposure is probably associated with an action of the corticosteroid. It has been reported that hydrocortisone can act directly upon the membranes of intracellular organelles, thereby inhibiting their release of membrane-limited protein molecules. Reports from a number of laboratories [36–39] have indicated that the granular fraction of leukocytes contains a cationic protein. This protein when released from its intracellular locus can initiate, by a yet unknown mechanism, a series of reactions terminating in the chemotactic attraction of leukocytes, as well as vasodilation and enhanced vascular permeability. The decreased peribronchiolar leukocytic infiltrate found in the lungs of our experimental mice may be related to inhibition, by cortisone, of the release of this cationic protein. Other findings compatible with the foregoing conclusion are that the leukocytic component of the Arthus phenomenon,[40] the hyperemic response to trauma,[41] and leukocytic margination [41] are all reported to be decreased in corticosteroid-treated animals.

Further evidence that cortisone might be stabilizing the membranes of the granular (lysosomal) fraction of leukocytes and/or macrophages was revealed in the experiments with trypan blue. These experiments were performed since they may be considered to assay indirectly the efects of a lysosomal (granular) factor. The biologic action of this fraction is also thought to be related to a cationic protein which has been reported by several laboratories.[36–39,42,43] In our studies with trypan blue, a dye which binds to proteins,[28] a markedly diminished concentration of the dye in the lungs was found in the cortisone-treated mice, in comparison to that found in the lungs of control mice, following spore inhalation. Conceivably corticosteroids can stabilize membranes of polymorphonuclear leukocytes and/or alveolar macrophages and thus inhibit the

release of factors which contribute to the host's response to an inflammatory stimulus. This granular (lysosomal) cationic protein has not been found in circulating polymorphonuclear leukocytes [38] although it is regularly found in polymorphonuclear leukocytes within exudates. Therefore, it is possible that the decreased trypan blue concentration found in the lungs of experimental mice following spore exposure might be intimately associated with the decreased concurrent peribronchiolar leukocytic infiltrate also found in these animals.

Evidence that cortisone does not act directly on the spores but rather on the host comes from earlier findings. In-vitro studies by Jefferson and Sisco,[44] using *A. niger,* as well as our own studies with *A. flavus* revealed that the addition of cortisone acetate to suitable culture mediums showed no enhancement in the germination or growth of the fungi.

Since cortisone administration induces a leukopenia,[31] it was important to determine whether this effect was influential in the enhanced susceptibility of cortisone-treated mice to fatal pulmonary aspergillosis. Since pretreatment with pertussis vaccine has been found to induce a marked leukocytosis,[32] control and cortisone-treated mice were pretreated with this vaccine and then exposed to spores. Although the cortisone-treated, exposed animals no longer were leukopenic, but rather had a leukocytosis, they had as high a mortality with pulmonary aspergillosis as did cortisone-treated, nonvaccinated animals. These results indicate that the quantitative alteration in circulating leukocytes was probably not of great importance. On the other hand, our studies suggest that qualitative rather than quantitative differences in phagocytosing cells are of importance.

Our cytologic studies of alveolar macrophages and spores obtained by tracheobronchiolar lavage of the lungs of control and cortisone-treated mice allowed analysis of several interesting qualitative alterations. First, there appeared to be a similar degree of early phagocytosis of spores by macrophages in both groups. However, early germination of spores was found in the lung washings of the experimental, but not of the control, animals exposed in vivo. The rate of germination was rapid and was similar to that observed with spores of *A. niger* in liquid medium without cells as reported by Yanagita,[45] as well as in some of our own in-vitro studies with *A. flavus*. Second, studies with alveolar macrophages of control and cortisone-treated mice incubated with spores in vitro revealed that although a similar number of spores were observed intracellularly within macrophages, a larger number of viable spores remained after incubation with experimental macrophages than after incubation with control macrophages. These findings indicate that the alveolar macrophages of cortisone-treated animals were not as compe-

tent as control macrophages in arresting growth and proliferation, as well as in killing spores of *A. flavus*. The results of Roberts[46] on both acquired and natural resistance to infection with the fungus *Dermatophilus congolensis* are consistent with our findings. In case of acquired or natural resistance to infection, the spores were ingested and prevented from germinating by macrophages. In contrast, in these species (sheep and guinea pig) susceptible to infection, the spores entered the macrophages but their subsequent germination was not inhibited. Also, our findings of rapid ingestion of spores by macrophages of experimental mice without arrested spore growth or digestion are compatible with experimental findings by others. That particle ingestion is not necessarily associated with rapid particle digestion has been shown by the ultrastructural studies of Leake and Myrvik.[47] These investigators studied alveolar macrophages of rabbits that were given intratracheal injections of *Mycobacterium smegmatis*. Although the bacteria were rapidly engulfed by alveolar macrophages, this phagocytosis was not associated with rapid formation of a distinct peribacillary space or a well-defined phagocytic vacuole. Even 5 days after exposure, some alveolar macrophages did not contain such phagocytic vacuoles surrounding the organisms.

Prior work by Cohn and Wiener[48] has shown that following phagocytosis the acid phosphatase activity of alveolar macrophages shifts from a predominantly particulate locus to the supernatant fraction of the phagocytosing macrophage. Also, studies of Weissmann[34] and Weissmann and Thomas[33] suggested that the pharmacologic effects of glucocorticoids might be at least partially due to the stabilization of lysosomal membranes by these steroids. By utilizing the findings of Cohn and Wiener,[48] it was possible to test whether the latter hypothesis[33,34] was applicable in our experimental model. Acid phosphatase was used as a "marker protein" for following a specific biochemical change within alveolar macrophages that has been thought to be related to phagocytosis. It was technically feasible to assay the activity of acid phosphatase in aliquots of homogenate and in its fractions prepared from alveolar macrophages obtained from lung washings. In control mice, acid phosphatase activity, following in-vivo spore phagocytosis was localized predominantly in the supernatant fraction of alveolar macrophages. In contrast, the activity of this enzyme was mainly localized within the particulate fraction of alveolar macrophages obtained from cortisone-treated mice. These findings are compatible with the concept that cortisone stabilizes lysosomal membranes and thereby may alter the reactions which normally ensue following phagocytosis. Actually our studies are the first demonstration at a cellular level that such a hypothesis may be applicable to experimentally induced infections in animals. Direct evidence that qualitative alterations of lysosomes in phagocytic cells may

be of importance in clinical diseases comes from the findings in Chediak-Higashi syndrome.[49-52] Patients with this syndrome have alterations of lysosomes in their leukocytes and exhibit a markedly enhanced susceptibility to secondary infection.

Summary

Earlier studies have demonstrated that cortisone-treated but not control mice develop a high incidence of lethal hyphal bronchopneumonia following a brief exposure to aerosols of spores of *A. flavus*. The present study was concerned with early morphologic and biologic responses in the lungs of cortisone-treated animals in an attempt to gain insight into the pathogenesis of the experimental infection. Within 9 hr. after spore inhalation, a decreased peribronchiolar leukocytic infiltrate was observed in lungs from cortisone-treated, as compared to control mice. Within 6 hr. spore germination was present in both alveolar macrophages and extracellularly in tracheobronchiolar washings obtained from cortisone-treated mice. In contrast, germination was rare in aspirates obtained from control animals. Acid phosphatase activity was found to be present mainly in the particulate fraction of alveolar macrophages obtained from experimental mice 8 hr. following in-vivo exposure to spores. In contrast, this enzyme was found mainly in the supernatant fraction of macrophages obtained from exposed control mice. In-vitro incubation of alveolar macrophages of cortisone-treated mice with spores revealed that more viable spores remained after incubation than after similar treatment using cells of control mice. Eight hours after spore inhalation and 45 min. following intraperitoneal trypan blue administration, the concentration of trypan blue bound to extravasated protein was decreased in the lungs of cortisone-treated animals as compared to controls. Increasing the circulating white blood cell concentration in mice by treatment with pertussis vaccine did not influence the high incidence of lethal hyphal bronchopneumonia in cortisone-treated mice following inhalation of spores. These data offer experimental evidence in support of the hypothesis that cortisone may act to stabilize lysosomal membranes within alveolar macrophages. This action influences the early cellular response in the lungs of experimental mice exposed to *A. flavus* spores. The altered qualitative response may be of major importance in the subsequent development of lethal hyphal bronchopneumonia.

References

1. Heffernan, A. G. A., and Asper, S. P., Jr. Insidious fungal disease: A clinicopathological study of secondary aspergillosis. *Bull Hopkins Hosp 118:*10–26, 1966.
2. Bodey, G. P., Powell, R. D., Jr., Hersh, E. M., Yeterian, A., and Freireich, E. J. Pulmonary complications of acute leukemia. *Cancer 19:*781–793, 1966.

3. CARBONE, P. P., SABESIN, S. M., SIDRANSKY, H., and FREI, E., III. Secondary aspergillosis. *Ann Intern Med 60:*556–567, 1964.
4. GRUHN, J. G., and SANSON, J. Mycotic infections in leukemic patients at autopsy. *Cancer 16:*61–73, 1963.
5. SIDRANSKY, H., and PEARL, M. A. Pulmonary infections associated with steroid and antibiotic therapy. *Dis Chest 39:*630–642, 1961.
6. BAKER, R. D. Leukopenia and therapy in leukemia as factors predisposing to fatal mycoses, mucormycosis, aspergillosis, and cryptococcosis. *Amer J Clin Path 37:*358–373, 1962.
7. HUTTER, R. V. P., and COLLINS, H. S. The occurrence of opportunistic fungus infections in a cancer hospital. *Lab Invest 11:*1035–1045, 1962.
8. KEYE, J. D., JR., and MAGEE, W. E. Fungal diseases in a general hospital: A study of 88 patients. *Amer J Clin Path 26:*1235–1253, 1956.
9. STRAATSMA, B. R., ZIMMERMAN, L. E., and GASS, J. D. M. Phycomycosis: A clinicopathologic study of fifty-one cases. *Lab Invest 11:*963–985, 1962.
10. TORACK, R. M. Fungus infection associated with antibiotic and steroid therapy. *Amer J Med 22:*872–882, 1957.
11. SYVERTON, J. T., WERDER, A. A., FRIEDMAN, J., ROTH, F. J., JR., GRAHAM, A. B., and MIRA, O. J. Cortisone and roentgen radiation in combination as synergistic agents for production of lethal infections. *Proc Soc Exp Biol Med 80:* 123–128, 1952.
12. MANKOWSKI, Z. T., and LITTLETON, B. J. Action of cortisone and ACTH on experimental fungus infections. *Antibiotics and Chemother (NY) 4:*253–258, 1954.
13. BAUER, H., FLANAGAN, J. F., and SHELDON, W. H. Experimental cerebral mucormycosis in rabbits with alloxan diabetes. *Yale J Biol Med 28:*29–36, 1955.
14. ELDER, T. D., and BAKER, R. D. Pulmonary mucormycosis in rabbits with alloxan diabetes; increased invasiveness of fungus during acute toxic phase of diabetes. *Arch Path (Chicago) 61:*159–168, 1956.
15. SCHOFIELD, R. A., and BAKER, R. D. Experimental mucormycosis (Rhizopus infection) in mice. The failure of chronic alloxan diabetes to modify host susceptibility. *Arch Path (Chicago) 61:*407–415, 1956.
16. ROTH, F. J., JR., FRIEDMAN, J., and SYVERTON, J. T. Effects of roentgen radiation and cortisone on susceptibility of mice to *Candida albicans. J Immun 78:* 122–127, 1957.
17. SIDRANSKY, H., and FRIEDMAN, L. The effect of cortisone and antibiotic agents on experimental pulmonary aspergillosis. *Amer J Path 35:*169–184, 1959.
18. LOURIA, D. B., FALLON, N., and BROWNE, H. G. The influence of cortisone on experimental fungus infections in mice. *J Clin Invest 39:*1435–1449, 1960.
19. SHELDON, W. H. and BAUER, H. The role of predisposing factors in experimental fungus infections. *Lab Invest 11:*1184–1191, 1962.
20. SIDRANSKY, H., and VERNEY, E. Experimental aspergillosis. *Lab Invest 11:* 1172–1183, 1962.
21. SIDRANSKY, H., VERNEY, E., and BEEDE, H. Experimental pulmonary aspergillosis. *Arch Path (Chicago) 79:*299–309, 1965
22. PIGGOTT, W. R., and EMMONS, C. W. Device for inhalation exposure of animals to spores. *Proc Soc Exp Biol Med 103:*805–806, 1960.
23. KIRCHSTEIN, R. L., and SIDRANSKY, H. Mycotic endocarditis of tricuspid valve

due to Aspergillus flavus, report of a case. *Arch Path (Chicago) 62:*103–106, 1956.

24. PINKETT, M. O., COWDREY, C. R. and NOWELL, P. C. Mixed hematopoietic and pulmonary origin of 'alveolar macrophages' as demonstrated by chromosome markers. *Amer J Path 48:*859–867, 1966.
25. COHN, Z. A., and WIENER, E. The particulate hydrolases of macrophages. I. Comparative enzymology, isolation, and properties. *J Exp Med 118:*991–1008, 1963.
26. GIANETTO, R. and DE DUVE, C. Tissue fractionation studies. 4. Comparative study of the binding of acid phosphatase, β-glucuronidase and cathepsin by rat-liver particles. *Biochem J 59:*433–438, 1955.
27. GOMORI, G. A modification of the colorimetric phosphorus determination for use with the photoelectric colorimeter. *J Lab Clin Med 27:*955–960, 1942.
28. JUDAH, J. D., and WILLOUGHBY, D. A. A quantitative method for the study of capillary permeability: Extraction and determination of trypan blue in tissues. *J Path Bact 83:*567–572, 1962.
29. GRIDLEY, M. F. A stain for fungi in tissue sections. *Amer J Clin Path 23:*303–307, 1953.
30. ASH, J. E. Technical methods in use at the Army Institute of Pathology. *J Tech Methods 26:*1–59, 1946.
31. DOUGHERTY, T. F., and WHITE, A. Influence of hormones on lymphoid tissue structure and function. The role of the pituitary adrenotrophic hormone in the regulation of the lymphocytes and other cellular elements of the blood. *Endocrinology 35:*1–14, 1944.
32. MORSE, S. I. and PICARD, M. B. Studies on the leucocyte response to B. pertussis in mice. *Fed Proc 23:*139, 1964.
33. WEISSMANN, G., and THOMAS, L. Studies on lysosomes. I. The effects of endotoxin, endotoxin tolerance, and cortisone on the release of acid hydrolases from a granular fraction of rabbit liver. *J Exp Med 116:*433–450, 1962.
34. WEISSMANN, G. Studies of lysosomes. VI. The effect of neutral steroids and bile acids on lysosomes *in vitro. Biochem Pharmacol 14:*525–535, 1965.
35. DE DUVE, C., WATTIAUX, R., and WIBO, M. Effects of fat-soluble compounds on lysosomes *in vitro. Biochem Pharmacol 9:*97–116, 1962.
36. JANOFF, A., SCHAEFER, S., SCHERER, J., and BEAN, M. A. Mediators of inflammation in leukocyte lysosomes. II. Mechanism of action of lysosomal cationic protein upon vascular permeability in the rat. *J Exp Med 122:*841–851, 1965.
37. ZEYA, H. I., SPITZNAGEL, J. K., and SCHWAB, J. H. Antibacterial action of PMN lysosomal cationic proteins resolved by density gradient electrophoresis. *Proc Soc Exp Biol Med 121:*250–253, 1966.
38. SEEGERS, W., and JANOFF, A. Mediators of inflammation in leukocyte lysosomes. VI. Partial purification and characterization of a mast cell rupturing component. *J Exp Med 124:*833–849, 1966.
39. JANOFF, A., BEAN, M. A., and SCHULLER, E. Mediators of inflammation in leukocyte lysosomes. III. Studies on lysosomes of rabbit peritoneal macrophages. *Life Sci 4:*2361–2372, 1965.
40. WARD, P. A. The chemosuppression of chemotaxis. *J Exp Med 124:*209–226, 1966.
41. ZWEIFACH, B. W., and KIVY-ROSENBERG, E. Microcirculatory effects of whole-body X-irradiation and radiomimetic procedures. *Amer J Physiol 208:*492–498, 1965.

42. GOLUB, E. S., and SPITZNAGEL, J. K. Dermal lesions induced by homologous PMN lysosomes. *Fed Proc 23:*509, 1964.

43. MOVAT, H. Z., URIUHARA, T., MACMORINE, D. L., and BURKE, J. S. A permeability factor released from leukocytes after phogocytosis of immune complexes and its possible role in the arthus reaction. *Life Sci 3:*1025–1032, 1964.

44. JEFFERSON, W. E., JR., and SISCO, G. The influence of exogenous steroids on the growth of *Aspergillus niger* and *Torula utilis. J Gen Physiol 44:*1029–1045, 1961.

45. YANAGITA, T. Biochemical aspects on the germination of conidiospores of *Aspergillus niger. Arch Mikrobiol 26:*329–344, 1957.

46. ROBERTS, D. S. The phagocytic basis of acquired resistance to infection with *Dermatophilus congolensis. Brit J Exp Path 47:*372–382, 1966.

47. LEAKE, E. S., and MYRVIK, Q. N. Digestive vacuole formation in alveolar macrophages after phagocytosis of *Mycobacterium smegmatis in vivo. J Reticuloendothel Soc 3:*83–100, 1966.

48. COHN, Z. A., and WIENER, E. The particulate hydrolases of macrophages. II. Biochemical and morphological response to particle ingestion. *J Exp Med 118:*1009–1020, 1963.

49. DENT, P. B., FISH, L. A., WHITE, J. G., and GOOD, R. A. Chediak-Higashi syndrome: Observations on the nature of the associated malignancy. *Lab Invest 15:*1634–1642, 1966.

50. WINDHORST, D. B., ZELICKSON, A. S., and GOOD, R. A. Chediak-Higashi syndrome; hereditary gigantism of cytoplasmic organelles. *Science 151:*81–83, 1966.

51. WINDHORST, D. B. Studies on a hereditary defect involving lysosomal structure, *Fed. Proc. 25:*358, 1966.

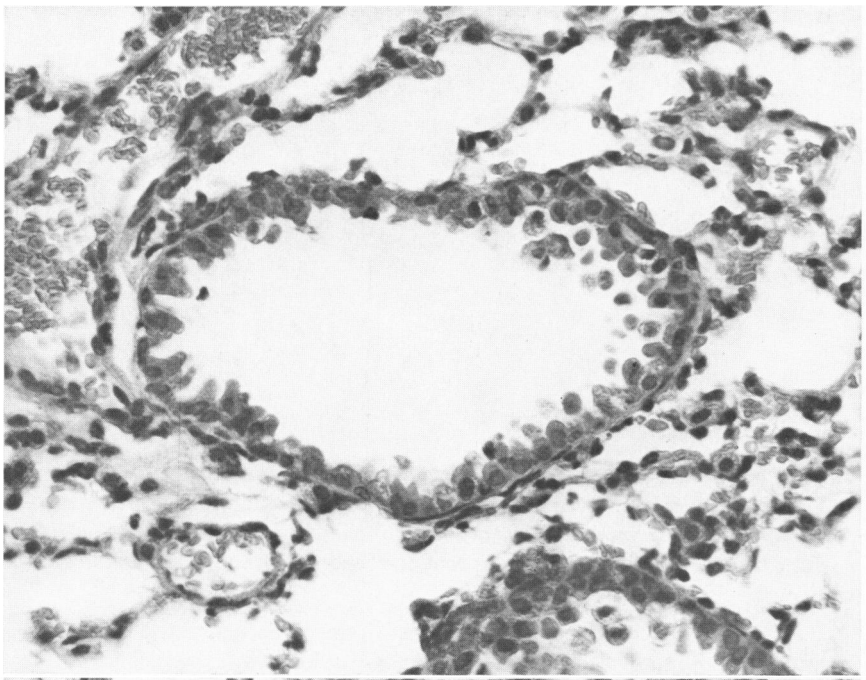
52. WHITE, J. G. Virus-like particles in the peripheral blood cells of two patients with Chediak Higashi syndrome. *Cancer 19:*877–884, 1966.

The skilled technical assistance of Miss Barbara Bartus is gratefully acknowledged, as is the assistance of Miss Elaine Geanopulos for her aid in preparation of the manuscript. The preparation of the illustrations by Mrs. LaRue Wosko and Miss Geraldine Morici is also appreciated. Dennis Falconer prepared the glass exposure chamber used in our studies.

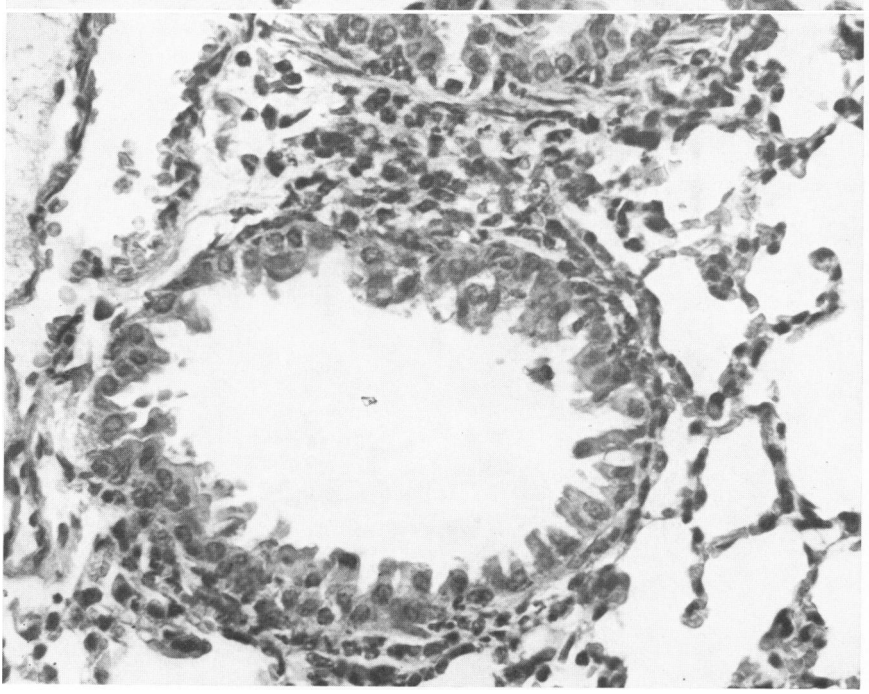
LEGENDS FOR FIGURES

FIG. 1. Lung from cortisone-treated mouse killed 9 hr. after exposure to *A. flavus* spores. Few inflammatory cells are present in peribronchiolar region. Hematoxylin and eosin stain. × 250.

FIG. 2. Lung from control mouse killed 9 hr. after exposure to *A. flavus* spores. Note localized peribronchiolar leukocytic infiltrate (compare with Fig. 1). Hematoxylin and eosin stain. × 250.



1

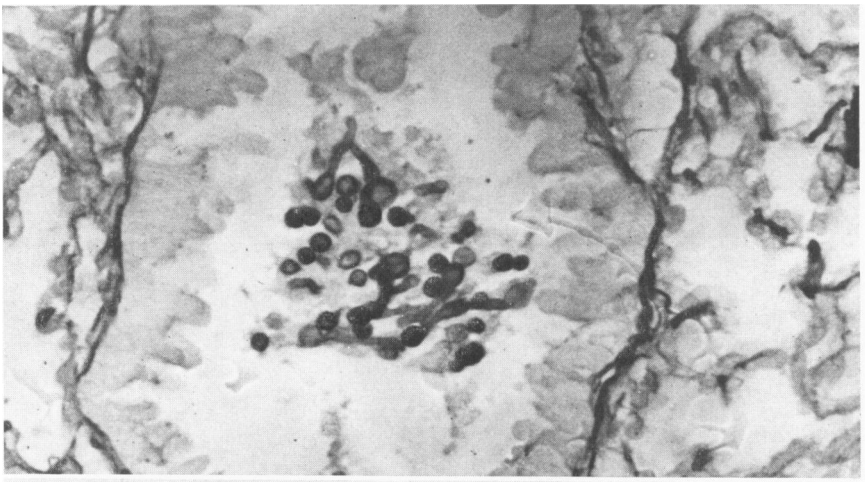


2

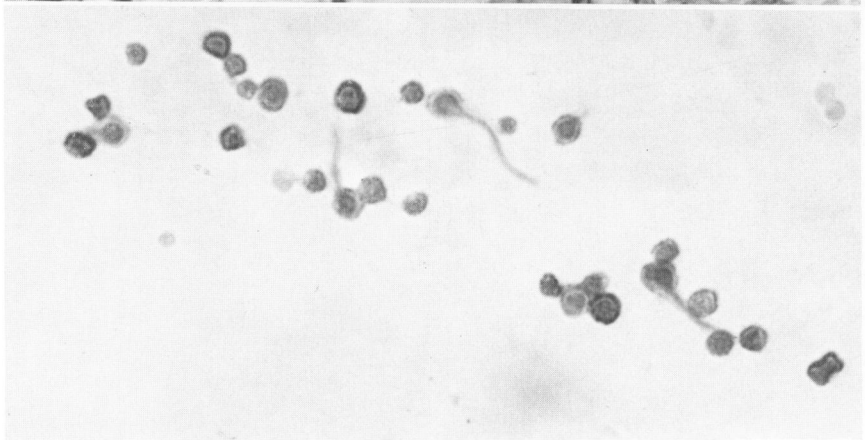
FIG. 3. Lung from cortisone-treated mouse 9 hr. after exposure to *A. flavus* spores. Germinating and nongerminating spores are present within lumen of bronchiole. In contrast, lung sections of control mice, similarly exposed and killed after same time as experimental animals contained spores but no evidence of germination. Gridley stain. × 400.

FIG. 4. Tracheobronchiolar washings obtained from cortisone-treated mouse 6 hr. after exposure to *A. flavus* spores. Spores of varying sizes as well as germ tubes are present. Sequence of events appears to be that small spores become enlarged preceding germ tube formation. Jenner-Giemsa stain. × 400.

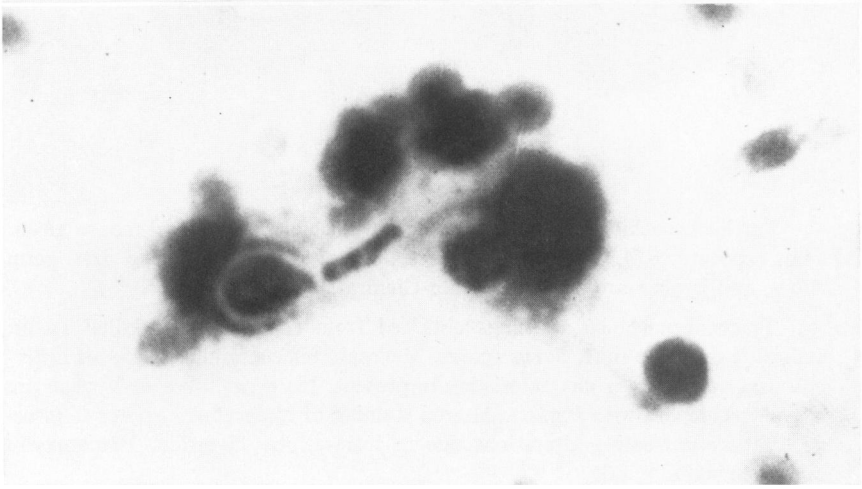
FIG. 5. Tracheobronchiolar washings obtained from cortisone-treated mouse 6 hr. after exposure to *A. flavus* spores. Germ tube originating from spore within alveolar macrophage is demonstrated. Nucleus of macrophage is seen adjacent to enlarged spore from which germ tube originates. Jenner-Giemsa stain. × 900.



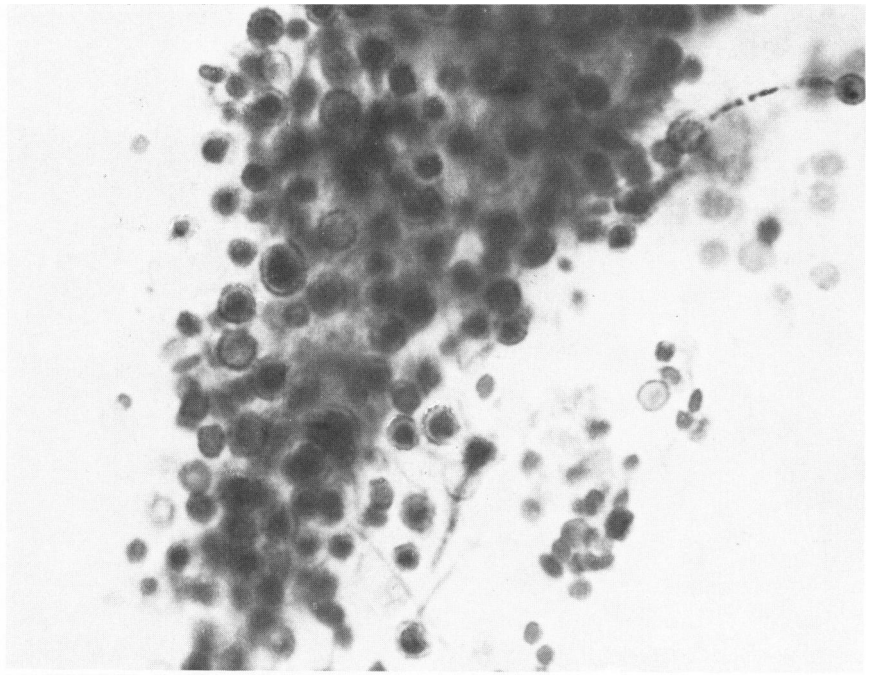
3



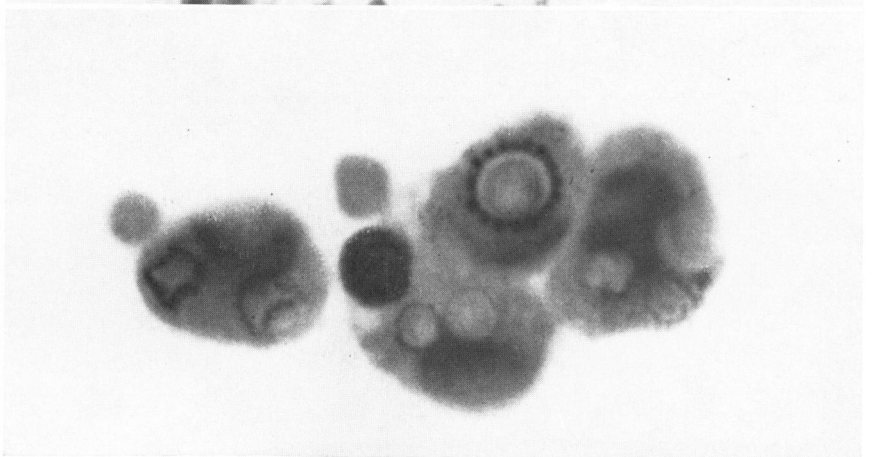
4



5



6



7

FIG. 6. Tracheobronchiolar washings obtained from cortisone-treated mouse 16 hr. after exposure to *A. flavus* spores. Collections of spores of varying sizes, germ tubes, and hyphae are present. Jenner-Giemsa stain. × 400.

FIG. 7. Tracheobronchiolar washings obtained from control mouse killed 16 hr. following exposure to *A. flavus* spores. Macrophages containing spores of different sizes and staining characteristic are present. No germ tubes or hyphae are present (compare with Fig. 6). Altered staining of intracellular spores is interpreted as representing alterations due to intracellular digestion. Hematoxylin and eosin stain. × 900.