INFECTION AND IMMUNITY, Aug. 1981, p. 575-582
0019-9567/81/080575-08$02.00/0

Vol. 33, No. 2

Primary Acute Histoplasmosis in Guinea Pigs Exposed to Aerosolized *Histoplasma capsulatum*

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Received 3 April 1981/Accepted 1 May 1981

Guinea pigs were examined as a possible in vivo model for human histoplasmosis. Guinea pigs were exposed to an aerosol of viable microconidia and mycelial fragments of *Histoplasma capsulatum* generated in a Henderson apparatus. Colonization and infection of the lungs occurred, with subsequent involvement of the regional lymph nodes and reticuloendothelial organs. Cultural recovery of the fungus from the nasopharynx and bronchoalveoli was initially high, but decreased with time. The mean number of colonies recovered from the lungs gradually increased, reaching a peak at 2 weeks, with involvement of the regional lymph nodes. Extrathoracic dissemination to the liver and spleen occurred in only a few animals. After 4 weeks, all tissues except the cervical and tracheobronchial lymph nodes were culturally negtive; all specimens were culturally negative after 8 weeks. Disappearance of *H. capsulatum* from tissues appeared to correlate inversely with the development of hypersensitivity as measured by skin test reactivity. Histopathological studies supported cultural results and were similar to those described for primary human and canine histoplasmosis.

Acute pulmonary histoplasmosis, acquired by inhalation of the spores and mycelial fragments of *Histoplasma capsulatum*, is a widely distributed endemic infection of man and animals. An asymptomatic or mild infection with skin test conversion, allergic manifestations, and spontaneous remission generally occurs more often than overt clinical disease (22). Involvement of the regional lymph nodes and hematogenous dissemination to the spleen and liver may occur (18, 24). Human infection may present a diverse and confusing spectrum of clinical manifestations, depending on the degree of exposure and the immunocompetence of the host (4).

The pathogenesis of experimental histoplasmosis (8, 25, 26) and development of histoplasmin sensitivity in animals after aerosol exposure have been previously described (13, 14). However, the organ distribution and pathway of dissemination after inhalation of *H. capsulatum* are not well documented. The present study defines the sequential pattern of primary acute histoplasmosis in guinea pigs after aerosol exposure as determined by cultural recovery, histopathology, and skin test reactivity.

MATERIALS AND METHODS

Inoculum. *H. capsulatum*, Scritchfield isolate, was maintained in the yeast phase on cystine heart hemoglobin agar (Difco Laboratories, Detroit, Mich.) slants at 35°C. Mycophil agar (BBL Microbiology Systems, Cockeysville, Md.) slants were inoculated with yeast cells, and the cultures were incubated at 28°C for 3 to 4 weeks for mycelial growth. This growth was suspended in saline (0.85%), and samples were added to flasks containing Smith sporulation agar. Five-week growth from this agar was scraped into 10.0 ml of sterile saline, and the suspensions from individual flasks were pooled. This suspension was homogenized in a water-jacketed Waring blender for six 10-s intervals and then centrifuged (10 × *g*, 10 min); the supernatant was decanted and placed in an ice bath. The sediment was resuspended in saline, filtered through 16-ply gauze, and centrifuged; the supernatant was pooled with the previous harvest. The final supernatant (adjusted to give a total particle count of 10^7 to 10^8/ml), which consisted of a suspension of microconidia and mycelial elements less than 10 μm, was used as the inoculum.

Experimental animals. Hartley strain guinea pigs (250 to 300 g) were obtained from Charles River Breeding Laboratories or from the breeding colony at the University of Oklahoma. Animals were separated by sex, kept in separate rooms at 22°C and a 12-h lighting cycle, distributed three per stainless steel cage, and allowed to acclimate 2 to 4 weeks before use. Animals were fed Purina Guinea Pig Chow once daily and were given distilled water supplemented with vitamin C (0.15 mg/ml) ad libitum. All guinea pigs were prebled and skin tested with histoplasmin before being used.

Aerosol exposure and sampling procedures.

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Unanesthetized guinea pigs (300 to 600 g) were exposed for 10 min to a fungal aerosol generated by a Collison nebulizer connected to a modified Henderson apparatus (7), followed by 1 min of sterile air. Total air flow through the apparatus was 28 liters per min, of which 8 liters per min were contributed by the nebulizer as droplet particles of 10 μm or less. Ambient temperature in the exposure chamber was 25°C, and 80 to 86% relative humidity was maintained. Two-minute aerosol samples were collected periodically in Porton glass impingers (Ace Glass, Inc., Vineland, N.J.) containing 10 ml of brain heart infusion broth (2.0 g of gelatin and 4.0 g of Na_2HPO_4 per liter) with an air flow of 12 liters per min. The spray factor and infectious dose were determined from impinger samples of the aerosol collected before, during, and after exposure of all animals. The infectious dose was computed on the basis of (aerosol concentration) × (duration of exposure) × (respiratory volume of the guinea pig at a breathing rate of 0.156 liters per min) (6). Viability determinations were made on fortified Sabouraud and mycosel agar (BBL) plates and incubated for 4 weeks at 24°C. After exposure, the facial surface of each guinea pig was wiped with Betadine solution or 70% alcohol, and the animals were returned to their cages. Noninfected control animals were either exposed to a saline aerosol or left untreated.

Experimental procedure. Individual guinea pigs of both sexes were randomly taken from their cages, injected intramuscularly with 1 mg of Benadryl (Parke, Davis & Co., Detroit, Mich.) 30 to 60 min before sacrifice (15), and weighed; the visceral body surface was shaved. Animals were exsanguinated by cardiac puncture, and blood was immediately cultured. Control animals of each sex were included at each necropsy period.

Nasal wash specimens were collected as follows: a catheter was inserted into the trachea with the lungs in situ, and 10 ml of Hanks balanced salt solution (BSS) was injected. Bronchoalveolar wash specimens were collected after removal of the lungs. The lungs were inflated with injections of two 20-ml volumes of Hanks BSS via a catheter inserted into the trachea. Approximately 10 to 15 ml was withdrawn from each wash and pooled. For the peritoneal wash, 30 ml of Hanks BSS was injected intraperitoneally, the external abdominal wall of the guinea pig was massaged gently, and the fluid was withdrawn. All washings were kept on ice, centrifuged (250 × *g*, 10 min), adjusted to give a total leukocyte count of 10^6/ml, and plated on fortified Sabouraud agar (1.0 g of yeast extract, 100 U of penicillin G per ml, and 100 μg of streptomycin per ml) and inhibitory mold agar (BBL).

Thoracic and abdominal organs, including the lymph nodes, were removed, and portions were placed in buffered Formalin; the remaining tissues were homogenized in glass grinding vessels with a Teflon pestle (Arthur H. Thomas, Philadelphia, Pa.) in Hanks BSS. Lymph nodes were kept separate (left and right) and cultured on blood agar containing gentamicin (40 μg/ml) and chloromycetin (25 μg/ml) and on mycosel agar. In addition, organ homogenates were plated onto fortified Sabouraud agar and inhibitory mold agar. Bone marrow cultures were not done.

Media. (i) Biphasic blood agar. Blood agar base (Difco) was prepared as directed. Sterile sheep blood (5%), gentamicin (20 μg/ml), and 0.05% sodium polyanethol sulfonate (Roche Diagnostics, Div. Hoffman-La Roche, Nutley, N.J.) were added to the cooled medium; 20 ml of this medium was dispensed into 60-ml Sani-glas bottles and allowed to solidify in a slanted position. Trypticase soy broth (2.5 ml; BBL) containing 0.05% sodium polyanethol sulfonate and gentamicin (20 μg/ml) was added to each bottle. An equal volume of blood was subsequently added (1:2 dilution), and the culture was incubated with the cap of the bottle left loose.

(ii) Trypticase soy broth. Trypticase soy broth with sodium polyanethol sulfonate and CO_2 (BBL 21480), or with agar surface (BBL 21472), was purchased commercially. The final blood-medium dilution was 1:10. Culture bottles were vented immediately with a sterile cotton-plugged 18-gauge needle.

(iii) Vacutainer blood culture medium. Vacutainer blood culture medium supplemented with peptone broth (BBL 4955) was used as directed. Final blood-medium dilution was 1:10. The venting unit was inserted after 24 h.

All blood cultures were incubated at 35°C in an atmosphere containing 5% CO_2 and were checked periodically for growth. Contents of the Vacutainer tubes were subcultured at 24 h onto fortified Sabouraud medium. After 5 weeks, all blood culture media were subcultured, and plates were held for 4 weeks at 22°C.

(iv) Hanks BSS. Calcium- and magnesium-free salt solution (Hanks BSS; GIBCO Laboratories, Grand Island, N.Y.) with gentamicin (20 μg/ml) was used for rinsing tissues; it served as the suspending medium for homogenizing organs. Heparin (10 U/ml) was added for nasal, bronchoalveolar, and peritoneal wash specimens.

(v) Smith sporulation agar. Smith sporulation agar was formulated by C. Smith, University of Kentucky, and contained 1.0 g of yeast extract and 20.0 g of agar per liter (adjusted to pH 6.5). After the agar was autoclaved, 2 ml of a mineral salt solution [20.0 g of $(NH_4)_2SO_4$, 6.0 g of K_2HPO_4, 1.0 g of $Na_3C_6H_5O_7 \cdot 2H_2O$, and 0.9 g of $MgSO_4$ per liter] was added.

Skin test. Guinea pigs were skin tested by the intradermal injection of 0.1 ml of histoplasmin 1:25 (Centers for Disease Control lot no. H42/68) and histoplasmin 1:100 (Parke, Davis) on the shaved visceral surface of each animal. Skin test reactions were read at 24 and 48 h by two different persons. Induration equal to or greater than 5 mm was considered positive. Guinea pigs of each sex were randomly selected for necropsy 5 days after being skin tested. This procedure was followed to prevent our utilizing only positive or negative reactors at any given period and to minimize any possible stimulating effect on serology.

Histopathology. Tissues from animals exposed to 1,748 viable fungal units (VFU) were fixed in 10% buffered Formalin, embedded in paraffin, sectioned at ≤5 μm, and stained with hematoxylin-eosin and Gomori's methenamine silver procedures (17).

RESULTS

Cultural recovery. Initially, two groups of 68 guinea pigs each were exposed either to 970

or 3,391 VFU of *H. capsulatum*. This experiment was performed to determine whether an approximately threefold increase in concentration would alter the distribution of the inoculum or would influence the number of guinea pigs infected or reverting to a positive skin test response. The results from the group of guinea pigs exposed to 970 VFU is expressed in Fig. 1.

Nasal and bronchoalveolar washings and the lungs yielded the earliest isolations of *H. capsulatum*. Nasal washings yielded the fungus through the first 2 weeks, but not thereafter. During the initial 5 weeks after exposure, bronchoalveolar washings were positive in 34 of 57 animals, and cultures from the lungs were positive in 14 of 57 animals. By 8 weeks, these specimens were culturally negative. *H. capsulatum* was not recovered from the spleen, liver, or blood at 8 weeks. During the 8-week period, total recovery from the spleen occurred in 4% (3 of 68), recovery from the liver occurred in 3% (2 of 68), and recovery from the blood occurred in 1% of the animals exposed to 970 VFU.

Similar results were obtained in the group of guinea pigs exposed to 3,391 VFU. Bronchoalveolar washings and lungs were culturally positive in 18 of 46 and 23 of 56 animals during the initial 5 weeks after exposure. Recovery from other organs during the 8-week period after exposure was as follows: spleen, 6 of 67 (9%); liver, 3 of 67 (4%); and blood, only 1%. All specimens were culturally negative by 8 weeks. The total number of culturally positive guinea pigs was 63% (43 of 68) for the group exposed to 970 VFU and 64% (43 of 67) for the group exposed to 3,391 VFU.

Cultures of *H. capsulatum* were obtained from after 2 through 8 weeks from the tracheobronchial and cervical lymph nodes. However, bacterial contamination prevented an accurate determination of the total number infected, and these results are not included. None of the guinea pigs died as a result of infection from the exposure procedure. No differences were apparent either in the distribution of the inoculum or in the number of guinea pigs infected.

In a second experiment, 89 guinea pigs were exposed to 1,748 VFU, and 65 of these animals were necropsied over a period of 8 weeks. The recovery of *H. capsulatum* from nasal and bronchoalveolar washings, lungs, and lymph nodes is presented in Table 1. *H. capsulatum* was recovered from all animals necropsied during the initial 4-week period after exposure, substanti-

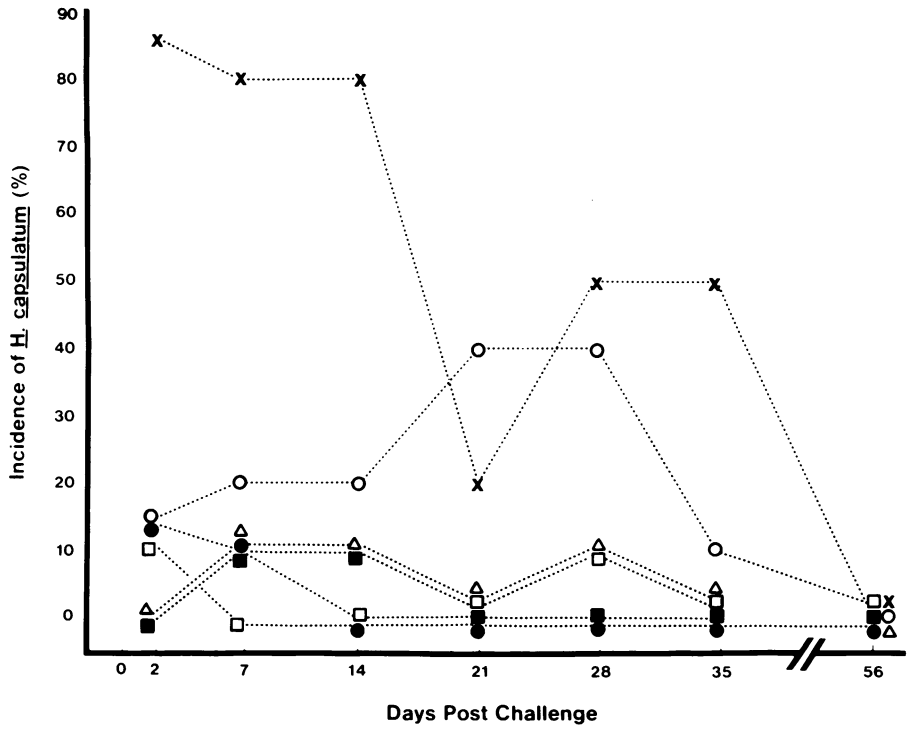

FIG. 1. *Isolation of H. capsulatum from guinea pigs exposed to 970 VFU. Symbols: ●, nasal wash; ×, bronchoalveolar wash; ○, lung; ■, liver; △, spleen; □, heart and blood.*

TABLE 1. *Recovery of H. capsulatum from guinea pigs exposed to 1,748 VFU*

Days postexposure	No. of guinea pigs necropsied[a]	Nasal wash		Bronchoalveolar wash		Lung		Lymph nodes[b]	
		% Positive[c]	Mean[d]	% Positive	Mean	% Positive	Mean	% Positive	Mean
0-2	6	67	66	100	36	100	2	0	0
3-7	6	0	0	100	3	67	1	33	1
14	4	50	<1	100	3	50	34	50	42
21	4	50	1	0	0	50	3	100	28
28	6	0	0	0	0	0	0	100	15
35	4	0	0	0	0	0	0	50	1
42	6	0	0	0	0	0	0	50	1
56	4	0	0	0	0	0	0	0	0

[a] Specimens from each guinea pig were cultured separately.
[b] Includes tracheobronchial and cervical lymph nodes.
[c] Percent positive guinea pigs.
[d] Mean colony number was calculated individually for each guinea pig (three plates per specimen) and then computed for all culturally positive animals necropsied at each time interval.

ating the effectiveness of the method of infection employed. Recovery dropped to 50% at 5 to 6 weeks, and all specimens were culturally negative 8 weeks after infection. The mean number of colonies recovered from the bronchoalveolar and nasal washings decreased within 48 h after exposure, becoming negative at 3 and 4 weeks, respectively. By 3 weeks, the lungs were positive in 50% of the animals necropsied, whereas all bronchoalveolar washings were negative. Lymph nodes were culturally positive 7 days after exposure, with the highest mean recovery of *H. capsulatum* at 2 weeks, the time at which peak mean recovery from the lungs was obtained and all animals had a culturally positive bronchoalveolar wash. By 3 weeks, *H. capsulatum* was recovered from the lymph nodes of all animals necropsied. The amount of growth recovered per node increased to peak at 2 weeks, the first time the tracheobronchial nodes were positive. Lymph nodes were positive in all animals at 3 and 4 weeks, although the mean recovery gradually decreased during this period. There was no recovery of *H. capsulatum* from the liver, spleen, heart, blood, kidneys, adrenal glands, thyroid, stomach, cecum, or appendix contents or from the axillary, mandibular, medial retropharyngeal, sternal, mediastinal, or intestinal (ileocolic, cecal, jejunal, or mesenteric) lymph nodes.

Delayed hypersensitivity. Guinea pigs first became skin test positive 2 weeks after exposure to all inoculum levels, and they generally gave maximum reactions at 3 to 4 weeks. Specifically, the following range of skin test reactions and millimeters of induration were recorded for individual guinea pigs exposed to 970 VFU and necropsied: 1 week, 0 of 17 positive, 2 weeks, 1 of 10 positive (6-mm induration); 3 weeks, 5 of 10 positive (6-, 7-, 10-, 11-, and 12-mm induration); 4 weeks, 7 of 10 positive (7-, 8-, 9-, 10-, 11-, 13-, and 14-mm induration); 5 weeks, 6 of 10 positive (8-, 8-, 9-, 9-, 10-, and 12-mm induration); 8 weeks, 9 of 11 positive (5-, 5-, 5-, 5-, 6-, 10-, 11-, 13-, and 15-mm induration). The guinea pigs exposed to 1,748 and 3,391 VFU showed nearly identical incidences of positive reactions and similar degrees of induration compared with those found with 970 VFU.

Serology. Serology performed on individual sera from guinea pigs exposed to 1,748 VFU and then necropsied demonstrated no complement-fixing antibodies to the yeast-form antigen during the initial 3 weeks after exposure. By 5 weeks, 100% of the animals necropsied had positive complement-fixing titers between 1:16 to 1:64, increasing to 1:128 by 8 weeks.

Histopathological examinations showed that lung lesions 7 days after aerosol exposure consisted of a widely disseminated, patchy bronchopneumonia in which macrophages, cellular debris, and a few polymorphonuclear leukocytes filled alveolar spaces and bronchial lumens (Fig. 2). Various numbers of intact, replicating, strongly Gomori's methenamine silver-positive *H. capsulatum* cells were demonstrated readily either outside of or within macrophages in the air spaces. The tissue-form cells of the fungus appeared as round to oval yeasts (2 to 5 μm in diameter) that produced single buds attached to the parent cell by a narrow base. In addition, single or clustered yeast-form cells appeared in submucosal nodules of bronchioles and in tracheobronchial lymph nodes. These latter yeasts were predominantly intracellular, and they stimulated a marked granulomatous inflammatory response that often obliterated the normal nodal architecture. At 14 days, lung lesions consisted of focal or diffuse interstitial widening by mono-

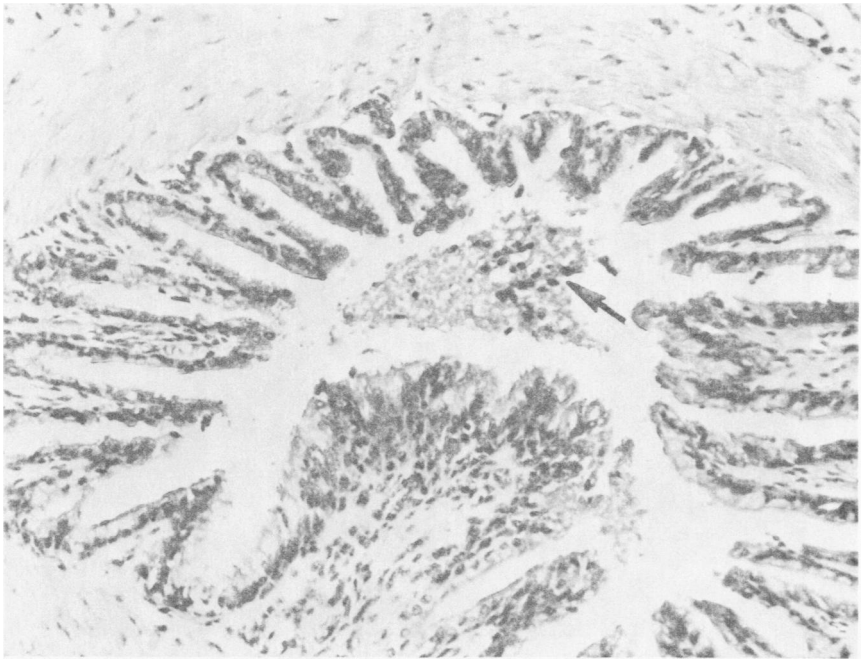

FIG. 2. *Yeast cells of H. capsulatum (arrow) mixed with inflammatory cell debris within the lumen of a bronchiole 7 days after aerosol exposure. Stained by Gomori's methenamine silver procedure and hematoxylin-eosin (17); magnification, ×150.*

nuclear inflammatory cells. Occasional yeast-form cells were present in these interstitial lesions. Fewer macrophages occupied the alveolar spaces in comparison to animals examined 7 days after exposure, and only an occasional macrophage contained *H. capsulatum* cells. Lymph node lesions were similar to those seen 7 days after exposure, but they were more severe and usually contained more yeast cells (Fig. 3). From 5 to 8 weeks, infiltration of the pulmonary interstitium by mononuclear cells and minimal involvement of air spaces were the usual findings. Yeast cells only rarely were demonstrated at this stage of the infection. In a few animals, the pneumonia appeared to have resolved completely. Moderate numbers of fungal cells were present in well-developed granulomas of the tracheobronchial lymph nodes in most animals. However, by 8 weeks after exposure only a few single and distorted yeast cells were demonstrated within the solid granulomas of these nodes. Neither *H. capsulatum* cells nor microscopic lesions were found in other organs at any time after aerosol exposure.

DISCUSSION

Cultural and histopathological examination demonstrated a sequential pattern in the clearance of *H. capsulatum* in guinea pigs after aerosol exposure. Generally, the fungus was cleared earliest from the nasal cavity in 2 to 3 weeks, probably by phagocytosis (9, 11) and mucociliary mechanisms. This clearance is suggested by the reduction in the mean recovery of colonies to one or less for the nasal washings and to three or less for bronchoalveolar washings from animals within the initial 48 h after exposure. In addition, the inhaled inoculum could have eluded the nonspecific defense mechanisms of the upper respiratory tract by lodging directly in alveoli and interstitial spaces. The percentage of animals with positive nasal washings also decreased during this interval. However, the lack of fungal recovery from all animals necropsied was probably a result of the absence of fungi in the upper respiratory tract or the recovery technique. During this same interval the bronchoalveolar washings were positive in all animals, although they showed the same decrease in mean colonies recovered, suggesting that *H. capsulatum* was generally not associated with, or did not colonize in the anterior portion of, the upper respiratory tract.

At the same time that the nasal washings were becoming negative, mean colonies recovered from the lungs increased, and the percentage of positive animals decreased. The mean number

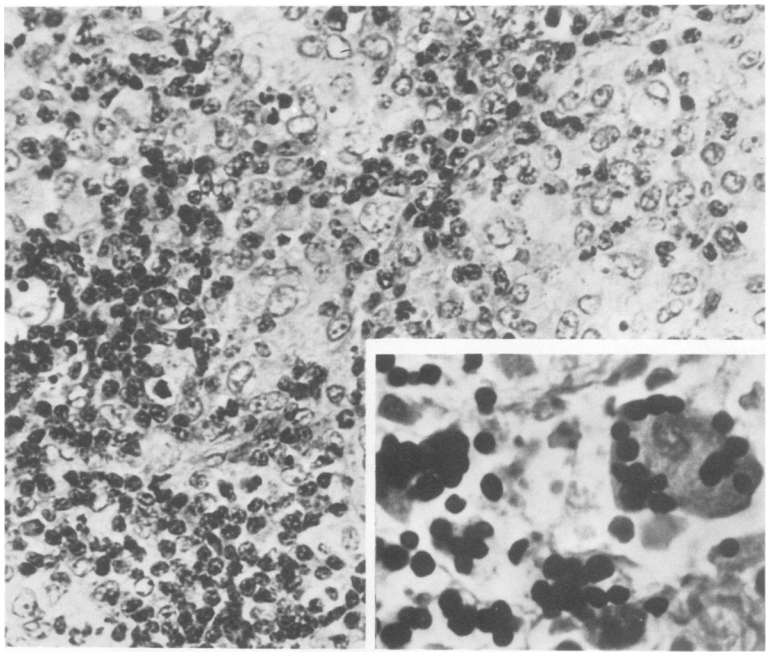

FIG. 3. *Tracheobronchial lymph node of guinea pig 14 days after aerosol exposure. Note that the nodal architecture is effaced by a marked granulomatous inflammatory response. Stained with hematoxylin-eosin; magnification, ×600. The inset shows the same area on a replicate section of the lymph node stained by Gomori's methenamine silver procedure and hematoxylin-eosin to demonstrate numerous yeast forms of H. capsulatum within epithelioid cells; magnification, ×600,*

of colonies increased from 1 by the end of 1 week to 34 at the end of 2 weeks, the highest for the entire study. These results are significant because only 50% of the animals were positive at this time although bronchoalveolar washings were positive for all animals necropsied. The parallel pattern displayed by the respiratory tract washings indicates the transitory nature of the fungus inoculum in the upper respiratory tract. The source of continued recovery after the initial 48 h most likely was the lung endogenously elaborating low numbers of yeast cells into the alveolar spaces. These results are supported by histopathology which demonstrated intact yeast cells free in the alveolar spaces 7 days after exposure. The recovery was not felt to be instrumental in this finding, since no tissue damage was noted in the alveolar epithelium.

Apparently, by 7 days the lymphatic system draining the lung and primary lung lesions transported the yeast cells to the regional lymph nodes, a time at which sufficient numbers were able to be detected by culture. The continual multiplication of *H. capsulatum* in the lung at this time resulted in increased yeast cells being recovered from the lymph nodes at 2 weeks. Our studies confirmed the report of *H. capsulatum* being able to survive phagocytosis by alveolar macrophages (3, 11). Only when the mean number of organisms reached a critical level did *H. capsulatum* escape into the bloodstream via the thoracic duct and disseminate extrapulmonary to the liver and spleen. Hematogenous dissemination to the liver and spleen is feasible because the cervical lymph nodes drain into the venous circulation and because it has been reported in other experimental animals (19, 20). This occurrence within the first week in the spleen and heart suggests that *H. capsulatum* evades the primary pulmonary defense mechanisms or lymphatics and is cleared in the spleen.

Straub and Schwarz (24) suggested that the presence of splenic calcifications is strong evidence of healed hematogenous dissemination in human and animal histoplasmosis, although isolation from the blood is uncommon. Our studies supported this finding.

This low recovery from the blood occurred despite the multiple blood culture systems used and the volume of blood cultured. The recovery of microorganisms from the blood is increased as the volume of blood cultured is increased (1). This low recovery suggests that dissemination is a sporadic event or that phagocytosis by polymorphonuclear leukocytes makes intracellular yeast cells unamenable to cultural recovery (10, 12).

Our inability to recover *H. capsulatum* except

from the cervical and tracheobronchial lymph nodes by 4 and 5 weeks (1,748 VFU) suggests that in the uncompromised, not previously exposed host, fungal units of *H. capsulatum* are adequately phagocytized and destroyed in the lung and peripheral organs (9, 10). If these lymph nodes are continuously receiving high numbers of organisms from the lung and multiplication of the yeast is occurring within the nodes, additional time is required for processing and elimination from these tissues. The success of this process is demonstrated by the gradual decrease after 2 weeks in total recovery from these lymph nodes which become culturally negative between 7 and 8 weeks after exposure.

Recruitment of the immune system occurred during this time. Skin test reactivity, first detected at 2 weeks, increased during the entire 8 weeks of sampling. Of the animals exposed to 1,748 VFU and necropsied from 4 through 8 weeks after exposure, 89% demonstrated complement-fixing antibodies. These results closely parallel those reported in human and animal histoplasmosis (2, 5, 24).

In humans, primary acute histoplasmosis may closely resemble acute infections produced by other mycological, bacterial, viral, and protozoal agents. Individual cases of acute histoplasmosis frequently are not recognized because of the nonspecific symptoms and the self-limited nature of the infection (16, 27). Primary infection occurs in a previously unexposed, uncompromised person when he inhales *H. capsulatum* which evades the nonspecific defense mechanisms of the respiratory tract and implants infectious units in alveoli and interstitial spaces (21). Growth of the fungus in the lung parenchyma may subsequently spread via the lymphatics and blood stream to the spleen, liver, and other organs before the host develops specific immunity (18, 23, 24).

The incubation period for a moderate to heavy inoculum requires a period of 1 to 3 weeks for development and detection of cellular immunity (5). An intense inflammatory reaction ensues at the initial foci of infection in the lungs, regional lymph nodes, and disseminated foci, leading to caseous necrosis. These necrotic lesions heal by fibrous encapsulation and calcification. Severity of the disease is dependent on initial inoculum and individual variability. The course of the disease is usually benign and self-limiting.

The true incidence of acute histoplasmosis is unknown. Frequently, *H. capsulatum* cannot be recovered in culture, detected serologically, or demonstrated histopathologically (24). This invariably requires that the diagnosis be made retrospectively.

The results reported herein detailing the cultural and histopathological aspects of *H. capsulatum* infection in the guinea pig closely parallel those observed in primary human and animal histoplasmosis, an acute primary pulmonary disease which is acquired by inhalation and which resolves spontaneously. These results explain in part why individual cases are difficult to recognize and usually are self limited and why determination of the true incidence of primary acute histoplasmosis as a clinical entity may never be possible.

ACKNOWLEDGMENTS

This study was supported in part by Public Health Service grant A1-00123 from the National Institute of Allergy and Infectious Diseases.

We thank Florence Deighton and Sharon Harris for their technical assistance and Rae Adams for assistance in preparing the manuscript.

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