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PATHOGENESIS OF EXPERIMENTAL HISTOPLASMOSIS IN GUINEA PIGS EXPOSED BY THE AIRBORNE ROUTE

DYNAMICS DURING PRIMARY INFECTION: CULTURAL STUDIES

A DISSERTATION

SUBMITTED TO THE GRADUATE FACULTY

in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

By

RONALD L. SCHLITZER

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APPROVED FOR THE DEPARTMENT OF

BOTANY AND MICROBIOLOGY

By

David H. Lamb

George C. Cozad

Donald C. Cox

J. B. Clark

Eddie Carol Smith

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PATHOGENESIS OF EXPERIMENTAL HISTOPLASMOSIS IN GUINEA PIGS EXPOSED BY THE AIRBORNE ROUTE

DYNAMICS DURING PRIMARY INFECTION: CULTURAL STUDIES

CHAPTER I

INTRODUCTION

Histoplasmosis is a granulomatous disease which characteristically results in a variety of clinical manifestations after inhalation of the spores or mycelial fragments of Histoplasma capsulatum, the etiologic agent. Primary infection results in a pulmonary inflammation that undergoes necrosis and becomes localized with subsequent encapsulation and calcification. This characterizes the benign or subclinical form of the disease. The lungs have been accepted as the natural portal of entry (9), and this route has been experimentally verified in mice (1) and dogs (2). In man, lung involvement has been indicated by roentgenographic and epidemiological evidence (17, 18). A common finding in the general pathology of the primary complex in histoplasmosis

has been the involvement of the regional lymph nodes (12, 13) and hematogenous dissemination as demonstrated by granulomas in the spleen and liver (14, 15) without obvious clinical illness.

Histoplasma capsulatum is a dimorphic fungus. Inocula from the mold form have been employed to experimentally infect different animal species by various routes, including intravenous (3), intraperitoneal (3,4,5), and intranasal instillation (4, 6). The significance of these routes to the more naturally acquired infection by inhalation has been discussed (7).

Larsh has demonstrated histoplasmin sensitivity in guinea pigs exposed to viable and killed mycelial particles (8) of H. capsulatum and reported the susceptibility of this species to infection by the airborne route (7).

The availability of the nose, nasopharynx, larynx and trachea to assault by H. capsulatum would seen obvious, yet clinical evidence indicates infection in these areas to be a rare occurrence (14). Interestingly enough, in light of all the clinical and experimental studies to date, the unequivocal pathway of infection following exposure to H. capsulatum remains unknown.

Previous observations in this laboratory demonstrated the cultural isolation of H. capsulatum from the lungs and tracheobronchial lymph nodes of guinea pigs exposed to either 970 or 3391 viable mycelial units (microconidia and mycelial

fragments) by the airborne route. Procknow (16) has reported similar results in dogs. The present study was planned to define experimentally the sequential pattern of infection after exposing guinea pigs to an aerosol of H. capsulatum. The sole criterion for infection was the isolation of H. capsulatum in culture from guinea pigs necropsied over a period of eight weeks after exposure in a Henderson apparatus.

CHAPTER II

MATERIALS AND METHODS

Fungus

Stock cultures of H. capsulatum, Scritchfield isolate, were maintained in the yeast phase on cystine heart-hemoglobin agar slants at 35 C and transferred three times weekly. Mycelial growth was obtained by transferring the yeast phase onto mycophil agar (BBL) slants and incubating at 28 C. Initial mycelial colonies were scraped and transferred to additional slants and incubated 3-4 weeks. This growth was scraped into sterile 0.85% saline. The suspension was decanted into a glass grinding vessel and homogenized for 10-20 seconds with a teflon pestle (Arthur H. Thomas, Philadelphia, Pennsylvania). Four milliliters of this homogenate were used to inoculate each of 16 flasks of Smith's sporulation medium.

Aerosol Inoculum Preparation

Mycelium was harvested at five weeks from 16 flasks of Smith's sporulation agar by scraping the surface of each flask into 10.0 ml saline with a rubber policeman. Each flask was rinsed with an additional 10.0 ml saline and pooled

with the previous harvest. The suspension was then homogenized in a water-jacketed Waring blender for six 10-second intervals, alternating with a 10-second rest period, for a total homogenization time of one minute. The homogenate was decanted into 50.0 ml screw-capped tubes and centrifuged for 15 minutes at 230 x g. The supernates were removed by pipette, pooled and stored in ice. The sediment was filtered through one 4 x 4 inch 16 ply gauze sponge (Johnson & Johnson). The filtrate was centrifuged for 15 minutes at 250 x g and the supernatant removed as above and pooled with the previous harvest. The concentration of this suspension was determined in a Neubauer hemacytometer. Microscopic observation indicated that all fungal units (mycelial and microconidia) were less than 10 microns. No macroconidia were observed. The suspension was adjusted to give a total particle count between 10^7-10^8/ml.

Experimental Animals

Hartley strain guinea pigs (250-300 g) were obtained from Charles River Breeding Laboratories (Wilmington, Massachusetts) or from the breeding colony at the University of Oklahoma. Animals were separated by sex in different rooms, distributed three/stainless steel cage, and allowed to acclimate 2-4 weeks before use. All animals were fed Purina Guinea Pig Chow once daily and given distilled water supplemented with vitamin C (0.15 mg/ml) ad libitum. They

were maintained under controlled conditions of temperature (22 C) and a 12-hour lighting cycle.

Media

Cystine heart-hemoglobin agar. Cystine heart agar and hemoglobin (Difco) were prepared as directed. After cooling, the sterile media were mixed and dispensed into 25 x 150 mm screw-capped tubes and slanted. This medium was used for maintenance of the yeast phase of H. capsulatum.

Mycophil (BBL). Commercially obtained medium was prepared as directed. The medium was dispensed into 25 x 150 mm screw-capped tubes, slanted, and used to convert H. capsulatum to the mycelial form.

Smith Sporulation Medium. The formulation was provided by Dr. Coy Smith (University of Kentucky, Lexington, Kentucky). In a two-liter Erlenmeyer flask 0.5 g yeast extract and 10.0 g agar were mixed in 500 ml distilled water, adjusted to pH 6.5 and autoclaved. After cooling, one milliliter of a mineral salt solution ($(NH_4)_2SO_4$, 20.0 g; K_2HPO_4, 6.0 g; $Na_3C_6H_5O_7 \cdot 2H_2O$, 1.0 g; $MgSo_4$, 0.9 g; distilled water, 1.0 liter) was added and the medium allowed to solidify.

Impinger Infusion Broth. The broth was prepared by dissolving 37.0 g Brain Heart Infusion (Difco); gelatin, 2.0 g; and Na_2HPO_4, 4.0 g in one liter distilled water and autoclaved. Ten milliliters were dispensed into each impinger to which four drops of sterile olive oil were added (dispensed

through a 26 gauge needle).

Hanks BSS-calcium and magnesium free (GIBCO). This basic salt solution was used to rinse tissues at necropsy. Gentamicin (20 mcg/ml) was added. This concentration was found to be non-inhibitory to H. capsulatum. Ten milliliters in each grinding vessel served as the suspending medium for homogenization. Heparin (10 units/ml) was added for nasal, alveolar, and peritoneal wash specimens.

Saline. NaCl, 8.7 g was dissolved in one liter distilled water and autoclaved.

Media for Cultural Isolation

All media used for cultural studies were dispensed with a Cornwall syringe into disposable 100 x 15 mm Optilux petri dishes (Falcon), 30-32 ml/dish.

Blood Agar. Blood agar base (Difco) was prepared as directed. To the cooled medium (50 C) the following components were added to yield the indicated concentrations: sterile sheep blood, 5% (Brown Laboratory, Topeka, Kansas); Gentamicin, 40 mcg/ml (Schering Corporation, New Jersey); Chloromycetin, 25 mcg/ml (Parke, Davis and Company, Michigan).

Fortified Sabouraud Agar. Neopeptone, 10.0 g; dextrose, 20.0 g; and yeast extract, 1.0 g, were dissolved in one liter distilled water to which 16.0 g agar were added. The medium was autoclaved for 15 minutes at 121 C. After cooling to 50 C, Potassium Penicillin G (E.R. Squibb and

Sons, Princeton, New Jersey) and Streptomycin sulfate (Pfizer Laboratories, New York) were added to give final concentrations of 100 units/ml and 100 mcg/ml, respectively.

Inhibitory Mold Agar (BBL). Commercially obtained medium was prepared as directed.

Mycosel (BBL). Commercially obtained medium was prepared as directed.

Media for Culturing Blood

Three media were used to culture the blood from each guinea pig.

Biphasic Blood Media. Blood agar was prepared as above except that Chloromycetin was not included. Instead, Gentamicin (20 mcg/ml) and 0.05% sodium polyanethol sulfonate (SPS) (Roche Diagnostics, New Jersey) were added. Twenty milliliters of this medium were dispensed into 60 ml Saniglas bottles and allowed to solidify in a slanted position. Trypticase soy broth (2.5 ml) and 0.05% SPS and Gentamicin (20 mcg/ml) were then added to each bottle. An equal volume of blood was subsequently added (1:2 dilution) and the culture incubated with the cap loose.

Trypticase Soy Broth. Trypticase Soy Broth with SPS, CO_2 BBL 21480) or with agar surface (BBL 21472) were purchased commercially. The final blood-medium dilution was 1:10. Culture bottles were immediately vented with a sterile cotton plugged 18-gauge needle.

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 hours. All blood cultures were incubated at 35 C in an atmosphere containing 5% CO_2 and checked periodically for growth. Subcultures were made from Vacutainer tubes at 24 hours onto fortified Sabouraud's medium. After 5 weeks, all blood culture media were subcultured and the cultures discarded. Subcultures were held for 4 weeks at 22 C.

Skin Test

Guinea pigs were skin tested by the intradermal injection of 0.1 ml Histoplasmin 1:25 (CDC Lot No. H42/68) and Histoplasmin 1:100 (Parke, Davis). Skin test reactions were read at 24 and 48 hours by two different persons. Induration equal to or greater than 5 mm was considered positive.

Aerosol Production and Sampling Procedures

Unanesthetized guinea pigs were exposed for 10 minutes to the fungal aerosol generated by a collison nebulizer connected to a modified Henderson aerosol apparatus (11) followed by a one-minute sterile air wash. The total air flow through the apparatus was 28 liters/minute of which 8 liters/minute were contributed by the nebulizer as droplet particles of 10 microns or less. Ambient temperature in the exposure chamber was 25 C, and 80-86% relative humidity was maintained.

Guinea pigs were returned to their cages immediately after exposure. An aliquot was withdrawn from the nebulizer suspension before and after all animals were exposed to determine total viability. Two-minute aerosol samples were collected in Porton all-glass impingers (Ace Glass, Inc., Vineland, New Jersey, #DWG B-2244) containing 10 ml of infusion broth, with an air flow of 12 liters/minute. Three impingers were used to sample the aerosol before, during, and after all animals were exposed, to determine the spray factor and infectious dose. The infectious dose was computed on the basis of aerosol concentration multiplied by the duration of exposure times the respiratory volume of the guinea pig, at a breathing rate of 0.156 liters/minute (20). Serial ten-fold dilutions were made in saline, of which 0.5 ml volumes were spread on 6 agar plates, 4 of each of fortified Sabouraud and 2 mycosel. Plates were incubated for four weeks at 24 C before colonies were counted and plates discarded.

Guinea Pig Necropsy

Individual guinea pigs of both sexes were randomly withdrawn from their cages after exposure. The weight of each was recorded and one milligram benadryl (Parke, Davis) was injected intramuscularly 30-60 minutes prior to sacrifice. After exsanguination by cardiac puncture, blood was immediately cultured, and an aliquot collected for serology. Aseptic technique was followed throughout necropsy. Initial-

ly, the axial, mandibular, retropharyngeal, and cervical lymph nodes, and the thyroid gland were removed. All tissues were trimmed of fat and left and right portions kept separate. Each was cut into three pieces, one of which was spread over, then pushed into the surface of a blood agar plate; another on a mycosel plate; the third piece was placed in buffered formalin. Nasal washings were obtained by inserting a catheter, attached to a 16-gauge needle, into the trachea and flushing the nasal cavity with 20 ml of Hanks BSS. This catheter was left in situ. The sternal and mediastinal lymph nodes were removed. The lungs and heart, still connected to the trachea, were dissected free of connective tissue and the esophagus and removed by cutting the trachea posterior to the incision made for the nasal wash catheter. An alveolar wash was obtained by flushing the lungs with two 20.0 ml volumes of Hanks BSS. These washings were pooled and maintained in ice. The heart was dissected free, minced and placed into a grinding vessel. The tracheobronchial lymph nodes were removed from the trachea and bronchi and cultured as were previous nodes. The trachea, mainstem bronchi, and portions of the secondary bronchi were trimmed from the lungs and placed in formalin. Individual lobes of the lung were minced and placed in a grinding vessel. The peritoneal wash was obtained by injecting 30 ml Hanks BSS intraperitoneally. A small incision was made in the peritoneum, a 5 ml syringe (perforated with holes along the bottom)

inserted, and the fluid collected. Then the peritoneum was dissected away and the liver, gall bladder, spleen, kidney, adrenals removed, rinsed, minced and put in separate grinding vessels. Portions of each tissue were placed in buffered formalin. The ileocolic, cecal, jejunal, and mesenteric lymph nodes were excised and treated as were the others. Contents of the stomach, cecum, and appendix were removed and portions of these placed in separate grinding vessels.

The three wash specimens were centrifuged at 1200 rpm for 10 minutes; the pellet was resuspended in 2.0 ml Hanks BSS and total white cells counted in a hemacytometer (on alveolar and peritoneal washes only). One-half milliliter was plated onto 4 media: one plate each of fortified Sabouraud, blood agar, IMA, and mycosel. A slide was made from each wash and kept for staining. The entire wash specimen was utilized.

All tissue specimens (except lymph nodes) were homogenized in the grinding vessels with a teflon pestle. One-half milliliter of each homogenate was plated onto three media: one plate each of mycosel, fortified Sabouraud, and IMA or blood agar. Cultures were incubated at 24 C and colonies counted after four weeks.

Experimental Procedure

Guinea pigs weighing 300-700 g were employed in this study which consisted of two separate experiments and four

separate aerosol exposures. Noninfected control animals were either exposed to a saline aerosol or untreated. Control animals constituted at least 10% of the animals for any given exposure.

The first experiment exposed 80 guinea pigs to either 3391 or 970 viable mycelial units. This was to establish a range lower than those previously reported from this laboratory (7). The second experiment employed 200 guinea pigs and consisted of 3 different aerosol exposures. Initially, 140 histoplasmin-negative guinea pigs (300-600 g) were exposed to 23 viable mycelial units in the Henderson apparatus. Immediately after exposure and continuing for three weeks, 34 animals (17 male, 17 female) were necropsied as previously described. All remaining guinea pigs were skin tested 25 days after the initial exposure.

The second part of the second experiment involved exposing 58 guinea pigs from the previous group and 22 new guinea pigs (all skin test negative) to an infectious aerosol containing 125 viable mycelial units. Sixteen animals were necropsied from one hour to four days after exposure. The remaining 64 animals in this group and those remaining from the first exposure group (48 animals) were skin tested again at 10 and 13 weeks.

In the final part of the second experiment 54 guinea pigs from the previous two exposure groups (400-700 g) and 36 new guinea pigs (not previously skin tested) were exposed

to an infectious dose of 1748 viable mycelial units. Immediately after this exposure, animals were necropsied over a period of eight weeks, as previously described. The 58 guinea pigs not included in the last exposure were excluded because they weighed more than 700 g, a size not amenable to the animal-holding tubes. Fifty of these guinea pigs (25 males, 25 females) were sacrificed after being skin tested, and 8 were kept as exposed control animals. Skin tests were repeated 8 weeks after the last exposure.

CHAPTER III

RESULTS

The data in Table I and II show the results from the first experiment in which guinea pigs were exposed to aerosols containing 970 and 3391 viable mycelial units of Histoplasma capsulatum. (Preliminary studies in this laboratory showed the Hartley strain guinea pig to be infected by H. capsulatum in this dosage range, 31). The earliest isolations of the fungus were obtained from the nasal and alveolar washings, and from the lungs. In addition, the alveolar washings and the lungs yielded positive isolations most frequently. Nasal washing cultures yielded the fungus through the seventh day (2 of 17 and 4 of 16 animals exposed to 970 and 3391 viable mycelial units respectively). However, these specimens were negative after two weeks and throughout the remainder of the experiment. Positive cultures were obtained from alveolar washings from 34 of 57 and 18 of 46 animals as shown in Tables I and II. During the first five weeks, the lungs were positive in 14 of 57 and 23 of 56 animals exposed to 970 and 3391 viable mycelial units. Nevertheless, by eight weeks all lung specimens were culturally negative. The percentage of posi-

TABLE I

Isolation of H. capsulatum from Guinea Pigs Exposed to 970 Viable Mycelial Units

	Number of Specimens Positive for H. capsulatum						
Post Exposure Time (Days)	Nasal Wash	Alveolar Wash	Lung	Liver	Spleen	Heart	Blood
0-2	1/7[a]	6/7	1/7	0/7	0/7	0/7	1/7
3-7	1/10	8/10	2/10	1/10	1/10	0/10	0/10
14	0/10	8/10	2/10	1/10	1/10	0/10	0/10
21	0/10	2/10	4/10	0/10	0/10	0/10	0/10
28	0/10	5/10	4/10	0/10	1/10	1/10	0/10
35	0/10	5/10	1/10	0/10	0/10	0/10	0/10
56	0/11	0/11	0/11	0/11	0/11	1/11	0/11
Total	2/68[b] 3% [c]	34/68 50%	14/68 21%	2/68 3%	3/68 4%	1/68 1%	1/68 1%
Control	0/15	0/15	0/15	0/15	0/15	0/15	0/15

[a] Number of guinea pigs from which H. capsulatum was cultured / Total number of guinea pigs in the necropsy group.

[b] Number of guinea pigs from which H. capsulatum was cultured / Total number of guinea pigs necropsied.

[c] Cumulative percent of positive animals.

TABLE II

Isolation of H. capsulatum from Guinea Pigs Exposed to 3391 Viable Mycelial Units

	Number of Specimens Positive for H. capsulatum						
Post Exposure Time (Days)	Nasal Wash	Alveolar Wash	Lung	Liver	Spleen	Heart	Blood
0-2	2/6[a]	4/6	4/6	0/6	1/6	0/6	0/6
3-7	2/10	6/10	3/10	1/10	2/10	0/10	0/10
14	1/10	0[b]	4/10	1/10	1/10	0/10	1/10
21	0/10	2/10	5/10	1/10	1/10	0/10	0/10
28	0/10	2/10	4/10	0/10	0/10	1/10	0/10
35	0/10	4/10	3/10	0/10	1/10	0/10	0/10
56	0/11	0/11	0/11	0/11	0/11	1/11	0/11
Total	5/67[c] 7%[d]	18/57 32%	23/67 34%	3/67 4%	6/67 9%	2/67 3%	1/67 1%
Control	0/15	0/15	0/15	0/15	0/15	0/15	0/15

[a] Number of guinea pigs from which H. capsulatum was cultured / Total number of guinea pigs in the necropsy group.
[b] Contaminated cultures.
[c] Number of guinea pigs from which H. capsulatum was cultured / Total number of guinea pigs necropsied.
[d] Cumulative percent of positive animals.

tive isolations of H. capsulatum at the designated necropsy periods suggested a pattern in the infectious process. Concomitant with the nasal washings becoming negative, the number of positive lungs increased with time, as did the alveolar washings. Nevertheless, both were negative by eight weeks. This pattern was more evident in those guinea pigs exposed to the low infectious inoculum. Recovery of the fungus from other organs was less than ten percent for the entire study. Positive cultures of H. capsulatum were obtained from the tracheobronchial and cervical lymph nodes at two weeks and throughout the duration of the experiment. However, bacterial contamination prevented an accurate determination of the total number infected. Therefore, these results were not incorporated into Tables I and II. Randomly selected guinea pigs were necropsied 5 days after being skin tested. This procedure was followed so as to prevent utilizing only positive or negative reactors at any given autopsy period. Conversion to a positive skin test in these animals is in agreement with previous rates reported from this laboratory (31).

In a second experiment guinea pigs were exposed to aerosol inocula containing fewer infectious particles than animals exposed in the first experiment. The rationale of decreasing the inoculum was to delay the initiation of infection and subsequent events, thereby allowing one to follow the disease process utilizing cultural methods.

Guinea pigs were exposed to either one, two, or three separate, or multiple aerosols generated in the Henderson apparatus. Animals exposed to the first, second, or third aerosols received infectious doses calculated to be 23, 125, and 1748 fungal units respectively. Information concerning this protocol is presented in Table III.

One hundred forty skin test negative guinea pigs in Group 1 were exposed to 23 mycelial units. Thirty-four animals were sacrificed over a period of 23 days after exposure. Histoplasma capsulatum was recovered from the nasal washings of only one male guinea pig that was necropsied thirty minutes after exposure. Cultures and wash specimens from the other animals were negative. The remaining 107 animals were skin tested 25 days after exposure, but no areas of induration were observed.

Eighty skin test negative guinea pigs in Group 2 were exposed to 125 viable mycelial units. Twenty-two of these animals were new stock. Fifty-eight were from Group 1. Sixteen animals (7 males, 9 females) were sacrificed over a period of 4 days (day 25-29 for animals from Group 1). Fifty percent of the animals necropsied were exposed to one aerosol (first or second) and fifty percent were exposed to both the first and second aerosols (Table II). Two additional male guinea pigs died during this period and were included in this study (18 animals total). Histoplasma capsulatum was not recovered from any of the eighteen guinea pigs. Of the

two guinea pigs that died, one had a cyst-like growth attached to its spleen. This same animal weighed one-half that of its cage mates. The other animal had its entire stomach occupying the right side of its thoracic cavity. Its weight was comparable to its cage mates. The remaining 62 animals were skin tested 10 weeks after exposure to the 125 mycelial fragments. Again, no areas of induration were observed at 24 or 48 hours.

Group 3 consisted of 89 guinea pigs which were exposed to 1748 mycelial units. This group included 39 new guinea pigs, never skin tested; 2 animals from Group 1, 1 animal from Group 2; 27 animals previously exposed in Groups 1 and 2, and 20 saline control animals from Groups 1 and 2 (Table III). Fifty-three animals were necropsied over a period of 8 weeks after exposure. One male guinea pig died 12 days after exposure (previously exposed to 23 and 125 viable mycelial units). Histoplasma capsulatum was recovered from the tracheobronchial lymph nodes and lung only. The remaining 41 animals (22 females, 19 males) were skin tested eight weeks after exposure. Twenty-two guinea pigs (54%) had positive skin tests.

Cultural recovery of the fungus from guinea pigs necropsied from Group 3 are presented in Table IV. Histoplasma capsulatum was recovered from the nasal washings, lungs, and alveolar washings for a period of 3 weeks after exposure. However, these specimens became negative after this time and

TABLE III

Protocol for Guinea Pigs Exposed to H. capsulatum in Experiment Two

Experimental Exposure Group	Infective Dose (Viable Mycelial Units)	Necropsy Interval (Days)	No. Guinea Pigs Exposed	No. Guinea Pigs Necropsied	Experimental Group Exposure Combinations [a]	No. Guinea Pigs Exposed	
						Male	Female
1	23	0-23	140	34	1	17	17
2	125	0-4	80	18	1	1	1
						3	4
					1&2	5	4
3	1748	0-56	89	53	3	12	9
					1&3	1	1
					2&3	0	1
					1,2&3	22	7

[a] Guinea pigs exposed to aerosol 1,2 & 3 or combinations of these aerosols received 23, 125 or 1748 viable mycelial units.

TABLE IV

Cultural Isolation of H. capsulatum from Guinea Pigs in Group 3 Exposed by the Airborne Route

Post Exposure Time (Days)	Specimens Positive for H. capsulatum									
	Nasal Wash	Alveolar	Mandibular LN[b]	RP LN[c]	Cervical LN	TB LN[d]	Lung	Liver	Spleen	Peritoneal Wash
0-2	9/11[a]	11/11	0/4	0/3	0/4	0/4	10/11	0/11	0/11	0/11
3-7	1/7	7/7	0/7	0/5	2/7	2/7	6/7	0/7	0/7	0/7
14	2/5	4/5	0/5	1/2[e]	3/5	4/5	4/5	0/5	1/5	1/4
21	4/8	5/8	2/8	2/5	6/8	8/8	7/8	1/8	4/8	2/8
28	0/7	0/7	0/7	0/2	7/7	4/7	0/7	0/7	0/7	0/7
35	0/4	0/4	0/4	0/4	2/4	1/4	0/4	0/4	0/4	0/4
42	0/6	0/6	0/6	0/6	3/6	2/6	0/6	0/6	0/6	0/6
56	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
Total	16/53	27/53	2/46	3/32	23/46	21/46	27/53	1/53	5/53	3/52
Control	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10

[a] Number of guinea pigs from which H. capsulatum was cultured / Total number of guinea pigs in the necropsy group.
[b] LN - Lymph Nodes
[c] RP - Retropharyngeal
[d] TB - Tracheobronchial
[e] The presence and number of lymph nodes vary among guinea pigs (19).

throughout the remainder of the experiment. Tracheobronchial lymph nodes were culturally positive at day 3 and cervical nodes at day 7 after exposure. Cultural isolation from these lymph nodes was obtained throughout the remainder of the study. Positive isolation of the fungus from the retropharyngeal lymph nodes occurred at day 14 and 21 and from the mandibular lymph nodes at day 21 only. The spleen was positive at day 14 and 21 and from the liver at day 21. The liver and spleen were never both positive in the same animal. Peritoneal washings were culturally positive at day 14 and 21. There was no recovery of H. capsulatum from the thyroid gland, adrenal glands, kidneys, heart, blood, stomach, cecum or appendix contents; or from the axial, sternal, mediastinal, and intestinal (ileocolic, cecal, jejunal, mesenteric) lymph nodes.

The number of isolations from each animal is presented in Table V. Histoplasma capsulatum was recovered from all animals necropsied in Group 3 over a period of 4 weeks after exposure. Recovery dropped to 50% at 5 and 6 weeks and all specimens were culturally negative at 8 weeks. The individual values indicate a pattern of recovery after exposure from the different organs in the guinea pig. Early recovery from the nasal washings, lungs, and alveolar washings led to involvement of the lymph nodes (days 3 and 7) and the spleen and liver (days 14 and 21). All tissues and washes (except the lymph nodes) became negative by 4 weeks and remained so for

TABLE V

Fungal Isolation from Guinea Pigs in Group 3 Exposed to H. capsulatum by the Airborne Route

Post Exposure Time (Days)	Specimen Combinations Positive for H. capsulatum												
	No. Isolations[a] / No. Animals	NW[c] AW[d] Lung	NW AW	AW Lung	NW,AW Lung LN[e]	AW LN Lung	NW Lung Spleen	AW LN Liver	AW	Lung LN	NW LN Lung	AW,LN Lung Spleen	LN
0-2	11/11	8/11[b]	1/11	2/11									
3-7	7/7			3/7	1/7	3/7							
14	5/5				1/5	1/5	1/5		1/5	1/5			
21	8/8						2/8	1/8		1/8	1/8	2/8	1/8
28	7/7												7/7
35	2/4												2/4
42	3/6												3/6
56	0/5												
Totals	43/53	8/11	1/11	5/18	2/12	4/12	3/13	1/8	1/5	2/13	1/8	2/8	13/25

[a] Number of guinea pigs from which H. capsulatum was cultured / Total number of guinea pigs in the necropsy group.

[b] Number of guinea pigs from which H. capsulatum was cultured from all the indicated specimens / Total number of guinea pigs in the necropsy group.

[c] NW - Nasal Wash

[d] AW - Alveolar Wash

[e] LN - Lymph Nodes (includes tracheobronchial, cervical,mandibular and retropharyngeal)

the remainder of the experiment. The lymph nodes were culturally negative at 8 weeks.

The positive results of the nasal and alveolar washings and the lung for H. capsulatum are recorded in Table VI. A consistent pattern was evident. The mean number of colonies isolated from the nasal washings decreased at 7, 14, and 21 days, although the percentage of positive animals remained about the same. The mean colony count from the alveolar washings decreased at day 14, then increased by day 21; yet the percent of positive animals continued to decrease at 7, 14, and 21 days. The lungs showed a marked increase in the mean colonies recovered at 7, 14, and 21 days, but the percent of positive animals with positive lungs remained relatively constant.

TABLE VI

Mean Recovery of H. capsulatum from Guinea Pigs in Group 3
Exposed by the Airborne Route

Post Exposure Time (Days)	Nasal Wash			Alveolar Wash			Lung		
	#P/#T[a]	%Positive[b]	Mean[c]	#P/#T	%Positive	Mean	#P/#T	%Positive	Mean
7	10/18	56	22	18/18	100	23	16/18	89	2
14	2/5	40	15	4/5	80	10	4/5	80	42
21	4/8	50	6	5/8	63	25	7/8	88	65

[a] Number of positive specimens / Total number of guinea pigs necropsied.

[b] Percent of culturally positive guinea pigs.

[c] Mean colony number was calculated individually for each guinea pig then computed for all culturally positive animals necropsied at each period.

CHAPTER IV

DISCUSSION

These experiments have established by culture isolation the colonization of H. capsulatum within the respiratory tract of the guinea pig and subsequent dissemination following aerosol exposure in the Henderson apparatus. The present experimental design was selected based on the clinical manifestations reported in human and canine histoplasmosis. Anatomy and epidemiology were closely correlated in this disease although previous studies by other investigators failed to fully document the complete infectious process. For example, in 102 dogs from Kansas and Missouri, only the histoplasmin skin test was studied, and was positive in 14 percent of these animals (21). In Virginia, however, 14 or 49 (29%) dogs had a positive skin test but 22 of 50 (44%) of these animals revealed positive tissues (22). In a later study by these same investigators, 145 of 397 (37%) dogs from the same area yielded positive peribronchial lymph nodes (23). Similar culture results from Kentucky (24) reported the absence of the organism in the spleen and bone marrow cultures from 300 dogs. Negative spleen cultures and roentgenographic findings

by Straub and Schwarz (12) was strong evidence that a natural resistance to hematogenous dissemination was present in dogs.

In human histoplasmosis, infected persons usually manifest spleen involvement (12). The primary histoplasmic complex in the adult is a dry caseous lesion with little or no calcification and limited lymph node involvement. In children, the primary healed lesions display extensive involvement of the lymph nodes (25).

Reports in the literature concerning experimental histoplasmosis by the airborne route were found to be scarce. No report has been documented which details the spectrum of events following aerosol exposure to H. capsulatum. In the present study, guinea pigs were exposed to 23 and 125 viable mycelial units. This inoculum was apparently handled well since only 1 colony was recovered from 1 of 52 animals exposed to these 2 inocula. None of the animals exposed developed hypersensitivity to histoplasmin when tested at 25 days, 10 and 13 weeks after aerosol exposure to H. capsulatum and would indicate that they were not infected. This interval has previously been found adequate for demonstrating delayed hypersensitivity (7). Since the viability of H. capsulatum may decrease during atomization (8) and may fall below 50% in some inocula during aerosolization (28), it was conceivable that guinea pigs used in these experiments may have received an antigenic dose of over twice the viable inoculum. Nevertheless, this would suggest that the inocula were below

the threshold level required to stimulate skin reactivity as detected by the intradermal inoculation of histoplasmin. The negative reactions of animals exposed to 23 or 125 mycelial units or both and tested at 25 days and 10 weeks (110 animals total) does not agree with earlier studies from this laboratory (8) which reported 85% conversion to histoplasmin sensitivity in guinea pigs exposed to 3 viable particles. The reason for this discrepancy has not been resolved. However, the animals could have again received sufficient killed inoculum to stimulate skin test response. Ritter (31) reported that the development of skin test reactivity in guinea pigs exposed to 100 killed mycelial particles was only 7% (1 of 14 animals) with a mean induration of 1.2 mm. Likewise, when animals were exposed 3 times to killed particles (a total of 28 million particles), only 2 of 12 animals (17%) developed induration greater than 5 mm. This would suggest that guinea pigs in the present study did not receive a sufficient inoculum to stimulate any skin test reactivity.

Studies exposing guinea pigs to 970, 1748, and 3391 viable mycelial fragments show a definite sequential pattern: the clearance of the nasal cavity of H. capsulatum by 2-3 weeks post exposure, and initial decrease of fungus colonies and then increase by 3 weeks (Table VI). In addition, the lung also follows this general pattern. The clearance from the nasal cavity may be due to phagocytosis, germination of the fragments in the epithelium or by mechanical clearance

by respiration. Invasion and infection of the nasal cavity does not correlate well with clinical studies since involvement of this area of the respiratory tract has been considered to be a rare occurrence (14). The high number of colonies initially recovered from the nasal and alveolar specimens (Table VI) probably reflects the transitory nature of the inoculum immediately following inhalation. The fungicidal capacity of the neutrophil and monocyte (29, 30) has been reported and certainly suggests that phagocytosis played an important role in the infectious process during the first two weeks after exposure. By two weeks, active lesions may spill over into the bronchi, thereby accounting for the increased recovery (at 3 weeks) from alveolar washings and lungs. The mean number of colonies recovered from the lung by 7 days (2) and 14 days (42) indicates a focus for subsequent involvement of the regional lymph nodes (tracheobronchial and cervical) which become markedly positive at this time. Lung involvement also could explain extrapulmonary involvement of the spleen and liver at 14 and 21 days, a finding common in clinical histoplasmosis (25).

The lymph nodes most commonly infected were the tracheobronchial and cervical. The cervical nodes received afferent drainage from the tongue and efferents from the head and neck nodes. The left and right efferent vessels from this node terminate in the thoracic duct and venous circulation, respectively. The tracheobronchial nodes lie along

the lateral surfaces of the bronchi. Afferent vessels include lymphatics from the lungs, heart, and medial tracheobronchial lymph nodes. Efferent vessels terminate in either the sternal, axillary, or mediastinal nodes.

The tracheobronchial nodes were first positive at 3 days (2 of 2 animals) and both animals had positive lungs and alveolar washings. The mean number of colonies recovered from the lungs at this time increased, suggesting that the source of inoculum for these nodes was the lung. The cervical lymph nodes were positive at day 7 (2 of 3 animals) and both animals had positive alveolar washings and 1 had a positive lung. However, the remaining tissues were negative. By day 14, the amount of growth recovered per node increased and marked the first time that retropharyngeal nodes were positive. The efferent vessels of the retropharyngeal nodes terminate in the cervical lymph nodes. In this case and the 2 animals at 21 days, the tracheobronchial and cervical nodes were positive. At 3 weeks, 2 animals had positive mandibular node cultures; these nodes also terminate in the cervical lymph nodes.

One could speculate that the infection spread via the lymphatics, but this would represent a retrograde infection from the cervical nodes, a fact not established nor reported in the literature. A more likely explanation is that these lymph nodes received the fungus in drainage from the infected organ. Since the lymph nodes may not receive adequate periph-

eral circulation, they would not be amenable to sufficient contact with phagocytic cell types. Therefore, the fungus would proliferate and, with time, account for the increased recovery observed. Since H. capsulatum tends to proliferate in a mass instead of individual cells, they would physically present a form more difficult to engulf than would individual cells. It would, therefore, take more time for this mass to be cleared and eliminated, a finding suggested in this study when recovery from these nodes decreased to fifty percent at 5 and 6 weeks. That these lymph nodes were not infected immediately after exposure was seen from the negative cultures early in the study. It was apparent that only when the mean number of organisms in the lungs reached a threshold level was H. capsulatum recovered from the tracheobronchial lymph nodes. Since the primary complex in histoplasmosis has been shown to be a caseous lesion, the physical size or the nature of the granuloma itself may preclude its development in the lymph nodes.

The recovery of H. capsulatum from the spleen and liver at 14 and 21 days was difficult to explain by lymphatic spread. Since Histoplasma capsulatum was rapidly phagocytized by monocytes and polymorphonuclear leukocytes, hematogenous dissemination by these cell types would appear feasible and because the cervical lymph nodes drain into the venous circulation. Reports in the literature allude to this pathway in human histoplasmosis (14) yet isolations from the blood

stream were not common. The presence of splenic calcifications was a definitive indication of healed hematogenous dissemination. Histoplasma capsulatum inoculated into animals by different routes yielded positive cultures from the liver and spleen yet was difficult to culture from the blood stream (6).

Of 2 guinea pigs with a positive peritoneal wash at 3 weeks, 1 had a positive liver and 1 a positive spleen, while both had positive nasal, alveolar washings and lung cultures. Animals necropsied at the same time, however, had substantially lower recovery from the lungs. The positive reticuloendothelial organs could have been the source of the positive peritoneal wash. However, during the same period, 3 different animals (1 with a positive spleen) had negative peritoneal washings.

The clearance of all viable organisms, except from the cervical and tracheobronchial lymph nodes by 4 weeks, would appear to be significant. Salvin (26) reported that guinea pigs inoculated intraperitoneally with yeast cells had the greatest number recovered from their tissues 2 weeks after inoculation, and the spleen was the tissue most readily infected. There was a gradual decline in the number of positive tissues per animal with time after exposure. Regardless of the route of inoculation, the fungus (yeast phase) disseminated most readily to the spleen and liver. Histoplasma capsulatum was isolated from tissue (spleen, liver, kidney,

blood) during the first 4 weeks after inoculation. The cultural results from our study agree with Salvin's findings as stated above and with increased complement-fixing titers of 64 (1 animal) and 256 (3 animals) by 6 weeks, whereas precipitins were negative. The increase in the number of skin test positive animals with time after exposure to H. capsulatum reached 70% by 4 weeks (Tables I and II). This could explain the disappearance of organisms from the tissues at this time.

Isolation of H. capsulatum from the appendix contents from 1 animal 6 hours after exposure probably represented ingestion of the inoculum. This was not difficult to perceive, based on the grooming habits of this species. The inoculum was ingested and probably eliminated through the intestinal tract at the time of necropsy. Procknow et al. (6) reported that direct inoculation of spores into the stomach of mice resulted in its recovery from only 1 of 30 mice over a 6 month period, yet colonic contents of all mice remained negative (6). Similar results have been reported by Straub and Schwarz (12).

The above studies have shown the sequential pattern of events following airborne exposure of guinea pigs to H. capsulatum. This sequence agrees with numerous reports in the literature based on epidemiologic, experimental, roentgenographic and anatomical studies observed in human and canine histoplasmosis. Although these studies tend to docu-

ment the pulmonary route as the primary route of infection in histoplasmosis, one must admit other routes of entry could be possible. However, they represent the exception (12).

CHAPTER V

SUMMARY

The early events of experimental histoplasmosis in guinea pigs following aerosol exposure with the mycelial phase of H. capsulatum has been described. Early recovery of organisms from the nasal and alveolar washings was believed due to the transitory nature of the aerosol inoculum immediately after exposure. Subsequent colonization and infection of the lungs occur with involvement of the regional lymph nodes and reticuloendothelial organs. Four weeks after exposure the infection cleared from all tissues except the cervical and tracheobronchial lymph nodes. By 8 weeks, all specimens were culture negative for H. capsulatum. The data presented closely parallel the pathology observed in primary infection in human and canine histoplasmosis. These studies experimentally document the nares as the portal of entry of airborne H. capsulatum. They also define subsequent events in the respiratory tract which follow inhalation of H. capsulatum.

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