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In Vitro Response of Alveolar Macrophages to Infection with *Coccidioides immitis*

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Alveolar macrophages obtained from rhesus macaques (*Macaca mulatta*) by bronchial lavage were observed to phagocytize endospores and arthrospores of *Coccidioides immitis*. When the macrophages were subsequently maintained in vitro, the phagocytized spores developed into spherules. There was no significant reduction in the viability of *C. immitis* after phagocytosis by macrophages from normal macaques, nor was killing induced by the addition of immune serum, complement, or lung lining material obtained from the bronchial lavage fluid. The inability of the macrophages to kill *C. immitis* may in part be explained by the observation that *C. immitis* appeared to inhibit fusion of the phagosomes containing the fungal spores with the lysosomes within the macrophages.

Coccidioidomycosis is the result of the inhalation of airborne arthrospores of *Coccidioides immitis*. Infection is common in areas endemic for coccidioidomycosis, indicating arthrospores are not immediately killed in vivo, but the host response may vary from inapparent infection to disseminated disease (12). Within the lung the arthrospores develop into large multicellular structures, spherules, which spread by the process of endosporulation. The severity of the host's response to infection with *C. immitis* may reflect the ability of the pulmonary defense mechanisms to kill the spores and stop development of the spherules. Since alveolar macrophages were important in pulmonary defense against infection, a study of their response to *C. immitis* in vitro is important to an understanding of the mechanisms of host resistance in coccidioidomycosis.

Phagocytized arthrospores have been shown in macrophages found in the lung washings of recently infected mice. As the infection progressed within these mice, spherules were found in subsequent lung washings. However, when the lungs of infected immunized mice were washed, there appeared to be fewer spherules than in the lung washings obtained from infected mice which had not been previously immunized (13). Beaman et al. demonstrated that T lymphocytes from immune mice are essential for the transfer of resistance to a lethal infection to a normal host. However, the effector cells which kill *C. immitis* in immune animals have not been clearly demonstrated.

The following report describes the response of alveolar macrophages from normal animals to endospores and arthrospores. The effect of serum containing antibody and complement activity on the phagocytosis and killing of *C. immitis* by alveolar macrophages was studied. Finally, the interaction occurring within the phagocyte between the phagocytized spores and lysosomes was described.

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MATERIALS AND METHODS

Animals. Adult *Macaca mulatta* weighing 8 to 10 kg were used as a source of alveolar macrophages. Radiological examination did not reveal any pulmonary lesions or abnormalities, and all of the animals were tuberculin negative. They were housed in individual cages at the California Primate Research Center and had not been exposed to *C. immitis*.

Bronchial lavage technique. Before the lavage, the animals were fasted 24 h. Then the animals were given 10 mg of ketamine hydrochloride per kg of body weight, and a local anesthetic, tetracaine hydrochloride (Cetracaine spray, Haver Lockhart, Kansas City, Mo.), was sprayed around the laryngeal area. The bronchial catheter, ballooned, 15 French (Rusch Inc., N.Y., N.Y.), was sterilized by immersion in Cidex (IPCO Hospital Supply, San Francisco, Calif.) for 15 min and then washed in distilled water and dried before use. The lavage was performed as described by Schwartz and Christman (14). A total of 240 ml of phosphate-buffered saline in six aliquots was washed in and removed. The lavage fluid recovered was centrifuged at 800 × *g* for 15 min, and the cell pellet was washed once in phosphate-buffered saline and resuspended in 25% fetal calf serum–25 mM HEPES (*N'*-2-hydroxyethylpiperazine-N'-ethane sulfonic acid)-buffered minimum essential medium (GIBCO Laboratories, Grand Island, N.Y.) before addition to eight-chambered Lab-Tek slides (Lab-Tek Products Inc., Westmont, Ill.) or cover slips in 25-cm^2 petri dishes. A 100-ml amount of the cell-free lavage fluid was centrifuged at 40,000 × *g* for 30 min, and then the pellet was resuspended in 4 ml of minimum essential medium to obtain concentrated lung lining material.

Cultures. *C. immitis* endospores were obtained

from D. Pappagianis, University of California School of Medicine at Davis. The cultures were grown in modified Converse medium (10), and the endospores were harvested after 60 h of incubation. Arthrospores harvested from slants of glucose-yeast extract agar were stored in distilled water until used. *Candida albicans* (human isolate) also was obtained from D. Pappagianis. The culture was maintained on glucose-yeast extract slants. The experimental inoculum was grown in tryptose soy broth, centrifuged, and resuspended in phosphate-buffered saline. Some of the above organisms were stained with lactophenol cotton blue, and the stained cells were counted in a hemacytometer to determine the total number.

Infection of macrophages. For the phagocytosis studies, the macrophages were resuspended to a dilution of 10^6 cells per ml, with 0.3 ml added per chamber of an eight-chambered Lab-Tek slide. Endospores were mixed 1:1 (vol:vol) with 50% immune *M. mulatta* serum with a complement-fixing antibody titer specific for coccidioidin of 1:128 or normal *M. mulatta* serum in minimum essential medium and held at 20°C for 30 min before infection. Then the macrophage monolayers were washed, and 0.3 ml of a 1:10 dilution of the endospore serum mixture in 25% fetal calf serum-minimum essential medium was added per chamber. After 4 h the macrophage monolayers were air dried, fixed in methanol-alcohol, and stained with Giemsa or periodic acid-Schiff stain (11). Four hundred macrophages were counted to determine the mean percent phagocytosis ± standard error of the mean.

For determination of the total colony-forming units recovered from the macrophages after infection, 5×10^5 macrophages were added per well of a 96-well flat-bottomed plate (Tissue Culture Cluster, Costar Cambridge, Mass.) or per chamber of a Lab-Tek eight-chambered slide. After incubation at 37°C, the macrophage monolayer was infected with approximately one organism per 10 macrophages. At the time of infection and 4 and 8 h postinfection, three samples of infected macrophage monolayers and the culture supernatants were immersed in distilled water for 10 min at 37°C, and the cells were scraped off. The final dilution of the combined culture supernatant and lysed cells, which was 1:100 in distilled water, was blended in Vortex mixer, and 0.1 ml was streaked on the surface of glucose–yeast extract plates or Trypticase soy agar plates (BBL Microbiology Systems). The plates were incubated at 37°C for 48 h for *C. albicans* and for 72 h for *C. immitis*.

In some experiments, the endospores or yeast cells were mixed 1:1 (vol:vol) with the lung lining material for 15 min before infection of the macrophages.

Electron microscopy. The macrophage monolayers on cover slips were washed at different time intervals after infection and fixed by addition of Karnovsky fixative (8) for 30 min at 20°C. Then the macrophages were scraped off the cover slips with a rubber policeman, and the cells were collected in tubes and centrifuged to form a pellet. The pelleted cells were processed routinely for embedding in epoxy resin, sectioning, and examination.

Lysosome staining with acridine orange. A 1-ml quantity of 10^6/ml of macrophages in 25% fetal calf serum-minimum essential medium was added per cover slip in a 25-cm^2 plastic petri dish. The cells were allowed to attach to the cover slip for 3 h. Then the monolayers were washed in balanced salt solution to remove serum components. Five micrograms of acridine orange per ml was added, and the cells were incubated for 10 to 15 min. The cover slips were washed free of acridine orange, and fresh media were added before infection with endospores or *Candida*. Three hours postinfection the cover slips were washed, mounted on slides, and examined for evidence of fusion of the orange-colored lysosomes with the phagocytized endospores or *Candida* as previously described (1). The number of infected macrophages and the number of macrophages containing phagocytized organisms stained orange were determined.

RESULTS

Infection of macrophage cultures. The majority of the cells obtained by bronchial lavage of normal rhesus macaques were macrophages. The examination of 100 cells on smears stained with Giemsa from 17 different bronchial lavage samples from 10 different animals revealed that 69.2 ± 2% (standard error of the mean) of the cells were macrophages, 27.2 ± 1.3% resembled lymphocytes, and 3.3 ± 0.5% were polymorphonuclear leukocytes. The mean number of cells obtained in a lavage was $12 \times 10^6 \pm 8 \times 10^5$. The glass adherent cells were maintained in culture for 3 or 24 h before infection with endospores or arthrospores. After infection some of the endospores and arthrospores appeared to be intracellular when observed by light microscopy. Confirmation of this observation was made by electron microscopy, as illustrated in Fig. 1a of an arthrospore within a macrophage. Thus, the alveolar macrophages readily phagocytized *C. immitis* endospores and arthrospores. Since immunoglobulins and complement may be present in the lung, it was of interest to know what effect antibody and complement had on the phagocytosis of *C. immitis*. As shown in Table 1, incubation of endospores with serum obtained from an infected rhesus macaque or fresh serum with complement activity did not enhance the subsequent phagocytosis of the endospores by macrophages.

After phagocytosis by the macrophages, the intracellular endospores or arthrospores gradually increased in size, and by 24 h postinfection immature spherules were present (Fig. 1b). Within 48 h after infection spherules were present in the macrophage cultures (Fig. 1c). After further incubation of the infected macrophages, there was a decrease in the number of surviving adherent macrophages, and a mixture of intracytoplasmic germinating spores and spherules was found. Part of the observed decrease in the number of macrophages in each culture may have actually reflected clumping of the infected

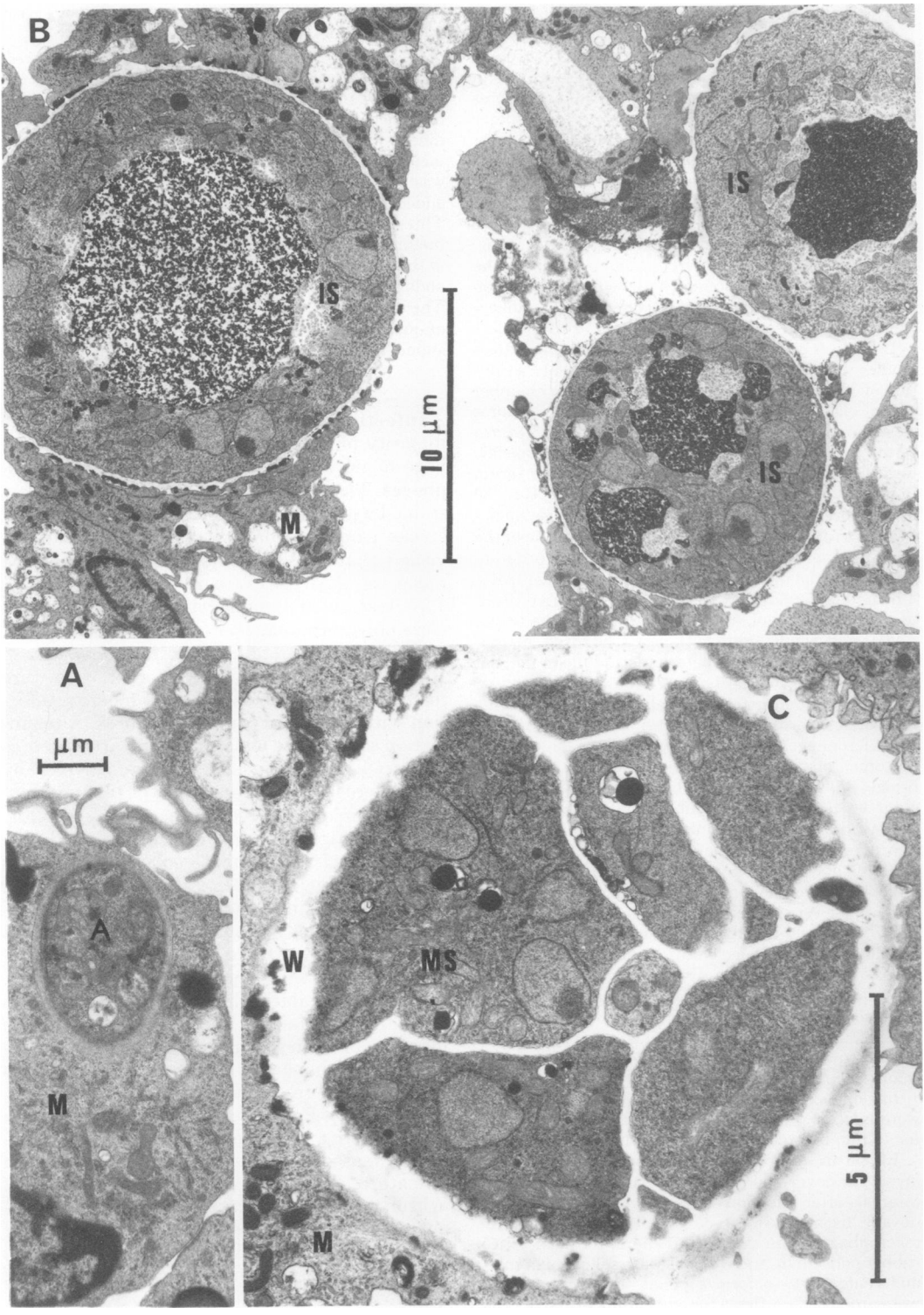

FIG. 1. *(a) Electron micrograph of an arthrospore (A) that has been phagocytized by an alveolar macrophage (M). (b) Electron micrograph of an immature spherule (IS) found after infection of an alveolar macrophage (M) with endospores. (c) Electron micrograph of a mature spherule (MS) with thick wall (W) found in an alveolar macrophage (M) 38 h after infection with endospores. The divisions within the spherule represent developing endospores.*

TABLE 1. *Effect of antibody and complement on the phagocytosis of endospores by alveolar macrophages*

Expt	Time cells cultured before infection (h)	Serum added	% Phagocytosis at 4 h postinfection
1	2	IMS[a]	19.8 ± 4.4[b]
		NMS	20 ± 4.5
2	2	IMS	15.8 ± 4
		NMS	24 ± 4.9
3	24	IMS	22.5 ± 4.7
		NMS	16 ± 4
4	24	IMS	19 ± 4
		NMS	18.8 ± 4
5	24	IMS + C[c]	14.5 ± 3.8
		NMS + C	10.3 ± 3.2

[a] The immune serum was obtained from a macaque with disseminated coccidioidomycosis (IMS). The complement-fixing antibody titer specific for coccidioidin was 1:128. The normal serum consisted of pooled samples from uninfected macaques (NMS) without detectable complement-fixing antibody specific for coccidioidin.

[b] The mean percent phagocytosis ± standard error was determined from observation of 400 cells.

[c] The complement (C) consisted of serum from normal macaques which was effective in facilitating hemolysis of antibody-coated erythrocytes.

macrophages or detachment of cells from the cover slip. A number of multinucleated giant cells similar to those seen in coccidioidomycotic lesions in vivo (Fig. 2) were seen in macrophage cultures 24 to 48 h after infection. Intracellular endospores were observed within the cytoplasm of some of the multinucleated macrophages, but it was not clear whether the giant cells could inhibit further development of the endospores to spherules.

Since it was important to know if the macrophages were fungicidal, the infected macrophages were lysed in distilled water, and the diluted lysate was plated on Trypticase soy agar at various time intervals postinfection. As shown in Table 2 there was little if any reduction in the number of colony-forming units of endospores or arthrospores recovered from the macrophages 4 h postinfection as compared to the colony-forming units recovered 10 min after infection. The incubation of endospores with immune serum or complement did not decrease the number of colonies of *C. immitis* recovered from the macrophages. Since there may be soluble factors within the lung which aid in macrophage function, the lavage fluid was concentrated by centrifugation and added to endospores before infection of macrophages. As shown in Table 3, the incubation of *C. immitis* endospores or a control organism, *C. albicans*, with lung lining material before infection of macrophage cultures did not enhance the fungicidal activity of the macrophages.

Phagosome-lysosome fusion. One explanation of the inability of macrophages to kill *C. immitis* may be that the spores inhibit fusion of the lysosomes with the phagosome within the macrophage. Since it was known that phagosomes containing *C. albicans* fuse with the lysosomes within macrophages (1), it was used as a control for similar studies of phagosome-lysosome fusion with *C. immitis*. After alveolar macrophages were incubated with acridine orange for 15 min, washed in phosphate-buffered saline, and infected with *C. albicans*, 69.8 to 76.9% of the infected macrophages contained orange yeast cells, indicating that fusion of the phagosomes containing yeast cells and the lysosomes containing acridine orange had occurred. In contrast, in only 20% of the macrophages infected with *C. immitis* could any fusion of the phagosome-lysosome be seen (Table 4). The difference was actually more dramatic in that five to six yeast cells in an infected macrophage would show fusion, but only one of several phagosomes containing endospores had fused with the orange-colored lysosomes. The addition of immune autologous serum to macrophages from a macaque which had recovered from a natural infection with *C. immitis* did not enhance the amount of phagosome-lysosome fusion observed (Table 4). After incubation with acridine orange, alveolar macrophages were washed and infected with arthrospores. In 78% of the control cultures infected with *C. albicans* phagosome-lysosome fusion had occurred; however, in 58.7% of the macrophages infected with arthrospores, phagosome-lysosome fusion had not occurred. Therefore, it appears endospores and arthrospores can inhibit phagosome-lysosome fusion in macaque alveolar macrophages.

DISCUSSION

The present investigation describes the infection of alveolar macrophages with *C. immitis* in vitro. Phagocytosis of both arthrospores and endospores was observed, but the addition of immune serum or complement did not enhance the uptake of *C. immitis*. This is in agreement with earlier observations by Beaman et al. (4) and Kong et al. (9) that the passive transfer of immune serum to mice did not prevent infection with arthrospores. The viability of the phagocytized endospores or arthrospores did not decrease after uptake by macrophages, and in fact spherules were subsequently observed 48 h after infection within clumps of macrophages. Apparently the alveolar macrophages from normal animals cannot destroy either endospores or arthrospores.

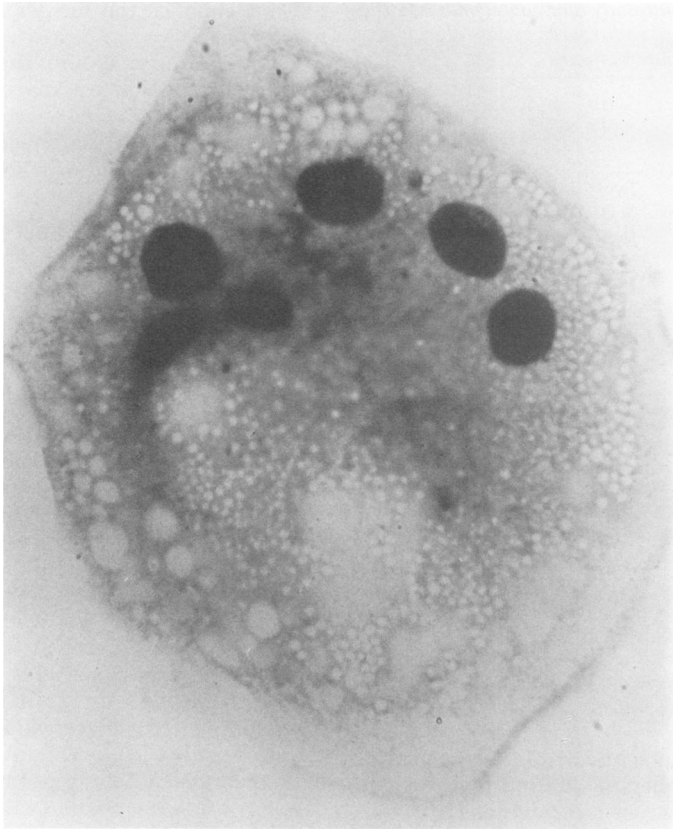

FIG. 2. *Light micrograph of a multinucleated macrophage found in an alveolar macrophage culture 48 h after infection with endospores (×1,000).*

TABLE 2. *Recovery of C. immitis after phagocytosis by alveolar macrophages*

Spores	Serum	Sample	CFU[a] recovered at time postinfection		
			10 min	4 h	8 h
Endospores	Fetal calf	Lysed cells[b]	14,066	16,833 ± 4,103	13,833 ± 3,719
		Inoculum[c]	18,333 ± 2,470	17,200 ± 650	14,667 ± 2,213
Endospores	Fetal calf	Lysed cells	9,850 ± 700	14,125 ± 820	17,050 ± 7,246
		Inoculum	9,500 ± 688	13,575 ± 583	15,653 ± 962
Arthrospores	Fetal calf	Lysed cells	10,750 ± 1,018	8,910 ± 972	
		Inoculum	13,000 ± 3,606	11,500 ± 3,391	
Endospores	IMS + C[d]	Lysed cells	34,970 ± 2,598	39,333 ± 3,135	
	IMS	Lysed cells	35,778 ± 2,990	35,783 ± 2,990	
	NMS + C′	Lysed cells	36,973 ± 3,040	35,223 ± 2,968	
	NMS	Lysed cells	31,083 ± 2,788	31,750 ± 2,818	

[a] CFU, Colony-forming units.

[b] Three infected macrophage samples were lysed in distilled water for 10 min, diluted, and plated on duplicate Trypticase soy agar plates. The counts are the mean colony-forming units ± the standard error of the mean obtained in four representative experiments.

[c] Samples from the inoculum used to infect the macrophages were handled in the same manner as the infected macrophages.

[d] The immune serum (IMS) had a complement-fixing antibody titer specific for coccidioidin of 1:128. The complement (C) was fresh serum from normal animals (NMS) which was effective in facilitating hemolysis of antibody-coated erythrocytes.

TABLE 3. *Recovery of C. immitis endospores and C. albicans from alveolar macrophages after exposure to lung lining material*

Organism	Lung lining material	Macrophages	CFU[a] recovered at time postinfection		
			10 min	1 h	2 h
C. albicans	Present[b]	Present	6,800 ± 477	4,900 ± 100	6,550 ± 578
	None	Present	5,500 ± 740	5,550 ± 373	5,450 ± 527
C. immitis	Present	Present	8,000 ± 447	5,600 ± 374	5,950 ± 335
	None	Present	6,150 ± 560	6,075 ± 339	4,450 ± 476

[a] The counts represent duplicate plates from each of two samples per time period. The mean colony-forming units (CFU) ± the standard error of the mean were determined.

[b] The infecting organisms were mixed with lung lining material 1:1 (vol:vol) before addition to macrophages.

TABLE 4. *Number of macrophages infected with C. immitis or C. albicans in which phagosome-lysosome fusion was observed*

Source of macrophages	Serum	Organism	No. of infected macrophages counted	% Infected macrophages with fusion[a]
Normal *M. mulatta*	Fetal calf	Endospores	149	20.8%
Normal *M. mulatta*	Fetal calf	*C. albicans*	156	76.9%
Normal *M. mulatta*	Immune[b]	Endospores	160	20.6%
Normal *M. mulatta*	Immune[b]	*C. albicans*	149	69.8%
Immune *M. radiata*	Fetal calf	Endospores	153	19.6%
Immune *M. radiata*	Autologous[c]	Endospores	133	19.5%
Normal *M. mulatta*	Fetal calf	Arthrospores	104	41.3%
Normal *M. mulatta*	Fetal calf	*C. albicans*	102	78.4%

[a] The number of infected macrophages showing fusion of the orange lysosomes with the phagosomes was counted.

[b] The serum was obtained from an infected *M. mulatta* and had a complement-fixing antibody titer specific for coccidioidin of 1:128.

[c] The autologous serum had a complement-fixing antibody titer specific for coccidioidin of 1:256.

The resistance of endospores to intracellular destruction may be dependent on the ability of *C. immitis* to inhibit phagosome-lysosome fusion. This had not been previously observed with *C. immitis*; however, *Mycobacterium tuberculosis*, *Toxoplasma gondii*, *Nocardia asteroides*, *Histoplasma capsulatum*, and *Chlamydia psittaci* have all been shown to inhibit fusion of the phagosome-lysosome in macrophages (5–7; C. Davis-Scibienski and B. L. Beaman, RES J. Reticuloendothel. Soc. **24**:29a, 1978). This ability of the organism may be one reason *C. immitis* can multiply in a normal animal. The phagocytosis of inhaled arthrospores by alveolar macrophages is probably of little initial benefit to the nonimmune host because the arthrospores or endospores readily grow and develop within the macrophages. Investigation is now under way to identify the effector cells in immune animals which can inhibit the development of spherules in the pulmonary tissue.

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