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Inhibition by Lysozyme of Growth of the Spherule Phase of *Coccidioides immitis* In Vitro

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Development of mature endosporulating spherules from an endospore inoculum was markedly inhibited by human or hen egg-white (HEW) lysozyme at 5 μg/ml. Mature spherules formed in medium containing 5 μg per lysozyme per ml (3.3×10^{-7} M) were approximately 50% smaller than control spherules. In addition, lysozyme induced a large portion of the endospore inoculum to revert to the mycelial growth phase. Increasing lysozyme concentrations to 10 or 20 μg/ml prompted a nearly complete reversion of the inoculum to the mycelial phase. Mature endosporulating spherules removed from growth medium and resuspended in a solution of human or HEW lysozyme at 18 μg/ml in distilled water prompted leakage of four to five times as much of materials absorbing maximally at 260 nm into the supernatant as untreated control spherules during 90 min of incubation. This four- to fivefold increase in nucleotide loss was evident at 4, 25, and 37 C. The permeability of 1-day-old immature spherules and 8-day-old endospores was considerably altered by lysozyme treatment of cells suspended in distilled water. Large amounts of potassium and nucleotides were rapidly lost by each type of cell when treated with 20 μg of lysozyme per ml. After 270 min of exposure to lysozyme, 98% of the immature spherules and 25% of the endospores were nonviable. Lysozyme adsorption by formalin-killed spherules in the presence of varying concentrations of calcium ion and the rapid alteration of permeability seen after lysozyme treatment suggested that the cell membrane was damaged as a result of binding lysozyme.

In a prior report (3) we described effects of hen egg-white (HEW) lysozyme on living and methiolate-killed spherules of *Coccidioides immitis*. At a concentration of 500 μg/ml, lysozyme markedly degraded walls of merthiolate-killed spherules in 3 days. *N*-acetyl glucosamine was solubilized, suggesting hydrolysis of chitin. Chitinase similarly degraded spherule walls although in considerably less time (16 h). Incubation of viable endospores and spherules in lysozyme solution resulted in loss of viability, whereas incubation in chitinase solution did not reduce viability suggesting that killing by lysozyme was unrelated to its enzymatic chitin-hydrolyzing activity.

In this present study we have attempted to quantitate the loss of some cytoplasmic materials from lysozyme-treated spherules. The effect of lysozyme on spherule morphogenesis was studied in medium containing concentrations of lysozyme similar to those present in normal human serum.

MATERIALS AND METHODS

Growth of spherules. The Silveira strain of *C. immitis* was maintained in the spherule growth phase using chemically defined modified Converse medium (MCM) according to the procedure of Levine et al. (10). In all experiments in which lysozyme was incorporated into the growth medium, Tamol-N, an anionic surface active agent (Rohm and Haas), was deleted because its inclusion caused the precipitation of lysozyme; lysozyme remained dissolved in MCM lacking Tamol-N (MCM-T). Spherules grown in MCM-T retained typical spherule morphology provided the endospore inoculum was grown in complete MCM. Spherules used in experiments to measure lysozyme adsorption were grown for 40 to 42 h in MCM-T and were killed by the addition of sufficient formalin to yield a final concentration of 0.18% HCHO followed by holding at 4 C for at least 8 days.

Nucleotide and potassium loss from spherules treated with human and HEW lysozyme. Human lysozyme (kindly provided by Elliot F. Osserman, The Institute of Cancer Research, College of Physicians and Surgeons, Columbia University, N.Y.) and HEW lysozyme (Sigma lot 72 C-8060) were solubilized at a concentration of 2.5 mg per ml of distilled water and were filter sterilized. All dilutions were made in distilled water except when otherwise noted. Human lysozyme was three times as active as HEW lysozyme in lysing *Micrococcus lysodeikticus* (Schwartz/Mann, lot V-3178). This difference in enzymatic activity between the two lysozymes has previously been noted (6, 11). Mature endosporulating spherules were grown

for 42 h in MCM-T. They were harvested by centrifugation at 1,500 × *g*, washed several times, and resuspended in distilled water at a concentration of 9.06 mg (dry weight) per ml. Triplicate tubes containing 9.0 ml of human lysozyme or HEW lysozyme at 20 μg/ml, or water only, were inoculated with 1.0 ml of washed spherules. One set each of tubes was incubated at 4, 25, and 37 C and agitated occasionally by hand. After 90 min the spherules were pelleted by centrifugation at 1,500 × *g*. The supernatants were examined in the range 240 to 300 nm with a Beckman DB-G spectrophotometer. An absorption maximum near 260 nm was taken to indicate leakage of nucleotides from spherules. Lysozyme in water at a concentration of 18 μg/ml absorbs less than 0.025 absorbance units at 260 nm; hence, lysozyme remaining unadsorbed to spherules contributed only slightly to absorbance by supernatants in this procedure. Yeast nucleic acid (Calbiochem, lot 4941) was used to provide a nucleotide reference spectrum.

The effect of cell age on lysozyme-induced loss of K^+ and nucleotides was investigated. Eight-day-old endospores in the stationary growth phase and 20-h pre-endosporulating spherules were harvested from MCM-T and washed several times with distilled water. The cells were resuspended at respective concentrations of 380 and 360 μg/ml in 15-ml volumes of 0 and 20 μg of HEW lysozyme per ml. The suspensions were incubated at 37 C for 270 min. At various times, 3.0 ml of suspension was removed after thorough mixing, and the cells were pelleted by centrifugation at 1,500 × *g*. The concentration of nucleotides in the supernatants was determined by ultraviolet spectrophotometry and the concentration of K^+ was determined by flame photometry. New plastic tubes were used throughout this experiment to minimize exogenous K^+ contamination. The viability of cells was estimated by exclusion of buffered methylene blue (5).

Influence of ionic strength on lysozyme uptake. Formalin-killed spherules were washed several times and resuspended in distilled water. A 1-ml amount of spherule suspension was added to tubes containing 5.0 ml of $CaCl_2$ at varying concentrations. Ca^{2+} was allowed to equilibrate with the spherules for 5 min. A 1-ml amount of HEW lysozyme solution was then added. The tubes were placed on a rotating tube drum (60 rpm) and incubated at 25 C. Lysozyme absorption by spherules was determined by decrease in supernatant absorbance at 280 nm after 15 and 45 min. Spherules allowed to bind lysozyme from aqueous solution for 1 h were pelleted by centrifugation and resuspended in equivalent volumes of $CaCl_2$, $MgCl_2$, NaCl, and water to determine if these cations could displace bound lysozyme.

Effects of HEW and human lysozyme and other polycations on the growth of spherules in vitro. Human lysozyme was added to 100 ml of MCM-T to yield final concentrations of 0, 5, 10, and 20 μg/ml. HEW was similarly employed at these concentrations and also at 50 and 100 μg/ml. Seven-day-old endospores were added until faint turbidity was just evident. After 2, 4, and 6 days of incubation, 5.0 ml of culture was removed and fixed with 0.5 ml of 37% HCHO. Turbidimetric monitoring of growth was determined by measuring the absorbance at 450 nm of formaldehyde-fixed suspensions. Prior to the taking of photomicrographs the cells were lightly stained with lactophenol cotton blue.

Alteration of normal spherule morphology and inhibition of growth by the presence of low concentrations of lysozyme might be due either to its enzymatic activity, i.e., hydrolysis of chitin or a surface-active effect. The latter would result from the large net positive charge of lysozyme at the pH employed (approximately 6.4). To discriminate between these two possibilities, spherules were cultured in MCM-T to which chitinase (Calbiochem, lot 201137) and the arginine-rich positively charged protein-protamine free base (Sigma, lot 121C-9510) had been added. Spherules were also cultured in the presence of the positively charged but nonpolymeric compounds spermine·4HCl (molecular weight 348.2), spermidine·3HCl (molecular weight 254.6), and putrescine·2HCl (molecular weight 161.1).

RESULTS

Temperature dependence of nucleotide leakage from lysozyme-treated endosporulating spherules. At each of the three temperatures employed (4, 25, and 37 C), the concentration of substances absorbing maximally at 260 nm was four- to fivefold greater in the supernatants of lysozyme-treated spherules than in the lysozyme-free controls (Fig. 1). At 25 and 37 C human lysozyme was approximately 25% more active on a weight basis in inducing nucleotide leakage than HEW lysozyme.

Loss of K^+ and nucleotides from lysozyme-treated stationary and log phase cells. Treatment of 20-h-old pre-endosporulating spherules and 8-day-old endospores with 20 μg of HEW lysozyme per ml resulted in considerable K^+ and nucleotide loss from each type of cell (Tables 1 and 2). Lysozyme clearly was more damaging to immature spherules than older endospores, for after 40 min only 10% of the spherules survived exposure to lysozyme, whereas there was no appreciable decline in endospore viability. After exposure for 270 min, 98% of the spherules were killed but only 25% of the endospores. Less damage to endospores by lysozyme was also suggested by their more gradual leakage of K^+ and nucleotides. Viability of control endospores and spherules was not significantly affected by incubation in distilled water lacking lysozyme. The morphology of spherules treated with lysozyme was consistent with their loss of cytoplasm (Fig. 2). Such cells stain readily with methylene blue.

Ionic strength and adsorption of lysozyme by formalin-killed spherules. Formalin-killed spherules (8.82 mg) adsorbed all the lysozyme (2.1 mg) from 7.0 ml of aqueous solution within 15 min. Similarly, lysozyme was completely

adsorbed in the presence of 7.1×10^{-5} and 7.1×10^{-4} M Ca^{2+} within 15 min. However, in the presence of 7.1×10^{-3} M Ca^{2+} only 52% of the lysozyme was adsorbed and, in the presence of 7.1×10^{-2} M Ca^{2+}, there was no adsorption of lysozyme by spherules. Washing cells which had bound lysozyme with 0.1 M Ca^{2+} or Mg^{2+} removed all bound lysozyme; however, washing with physiological saline (0.15 M Na^{+}) removed only 40% of bound lysozyme. Washing with water did not release bound lysozyme.

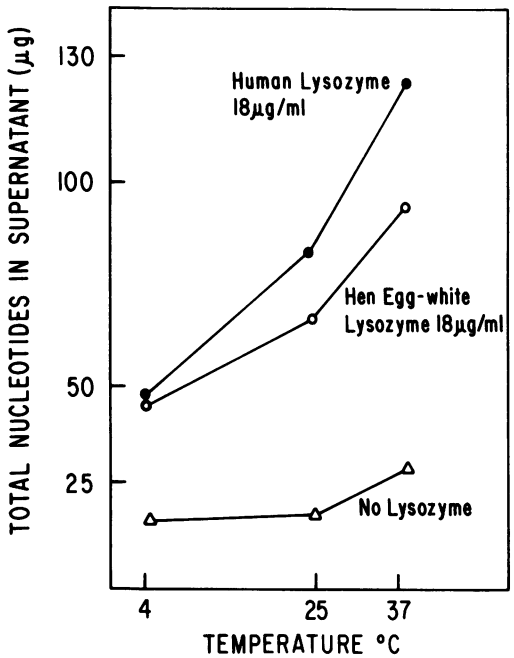

FIG. 1. *Nucleic acid loss after a 90-min exposure of 9.06 mg of mature spherules to lysozymes.*

Effect of human and HEW lysozyme on the growth of spherules in vitro. From an endospore inoculum adjusted to just vanishing turbidity, spherule growth in the MCM-T control flask reached a 450-nm absorbance of 0.215 after 42 h of incubation. After 42 h of incubation, flasks containing 5, 10, and 20 μg of human lysozyme per ml achieved absorbances of only 9.8, 3.7, and 2.8%, respectively, the absorbance of the lysozyme-free control. Flasks containing HEW lysozyme at the same concentrations showed absorbances of 6.5, 4.2, and 4.2% the value of the control. After 4 days of incubation, growth was apparent in all flasks containing lysozyme, but because it was largely mycelial, turbidimetric measurements were not done.

The Silveira strain of *C. immitis* after 42 h of incubation in lysozyme-free MCM-T produced large numbers of endosporulating mature spherules 25 to 35 μm in diameter (Fig. 3, left). Increasing the incubation period resulted in dispersion of endospores 2 to 4 μm in diameter which after 4 or 5 days increased in diameter to 6 to 10 μm (Fig. 3, right). No further development of endospores occurred until they were subcultured into fresh medium. This normal sequence of events was considerably altered by the incorporation of 5 μg of human lysozyme per

TABLE 1. *Loss of K^+ and nucleotides from lysozyme-treated spherules*

Time (min)	No treatment			Lysozyme (20 μg/ml)		
	K^+	Nucleotides	Viable cells (%)	K^+	Nucleotides	Viable cells (%)
40	0.07[a]	0.46	95	1.36	5.22	10
100	0.10	0.99	95	1.43	5.71	7
180	0.16	1.52	95	1.54	6.16	7
270	0.20	1.81	95	1.54	6.32	2

[a] Milligrams lost per 100 mg of cells (dry weight).

TABLE 2. *Loss of K^+ and nucleotides from lysozyme-treated endospores*

Time (min)	No treatment			Lysozyme (20 μg/ml)		
	K^+	Nucleotides	Viable cells (%)	K^+	Nucleotides	Viable cells(%)
40	0.07[a]	0.32	95	0.87	1.18	95
100	0.08	0.39	95	1.13	1.44	90
180	0.08	0.53	95	1.29	1.80	80
270	0.08	0.64	92	1.39	2.10	75

[a] Milligrams lost per 100 mg of cells (dry weight).

ml of MCM-T. After 42 h no mature spherules were present; instead immature spherules 12 to 18 μm in diameter and inoculum endospores either unchanging or reverting to the mycelial phase were seen (Fig. 4, left). After 4 days, mycelial growth was quite extensive and some small endosporulating spherules, 16 to 24 μm, were noted (Fig. 4, right). Human lysozyme at 10 μg/ml strongly inhibited growth after 42 h of incubation. After 4 to 6 days an intermediate type of morphology between the spherule and mycelial phase was seen. Chains of cells suggestive of chlamydospores were abundant. Many of the chlamydospore-like cells revealed a close relationship to spherules by cleaving into endospores (Fig. 5, left). Human lysozyme at 20 μg/ml induced a nearly complete reversion to the mycelial phase. Structures resembling bar-

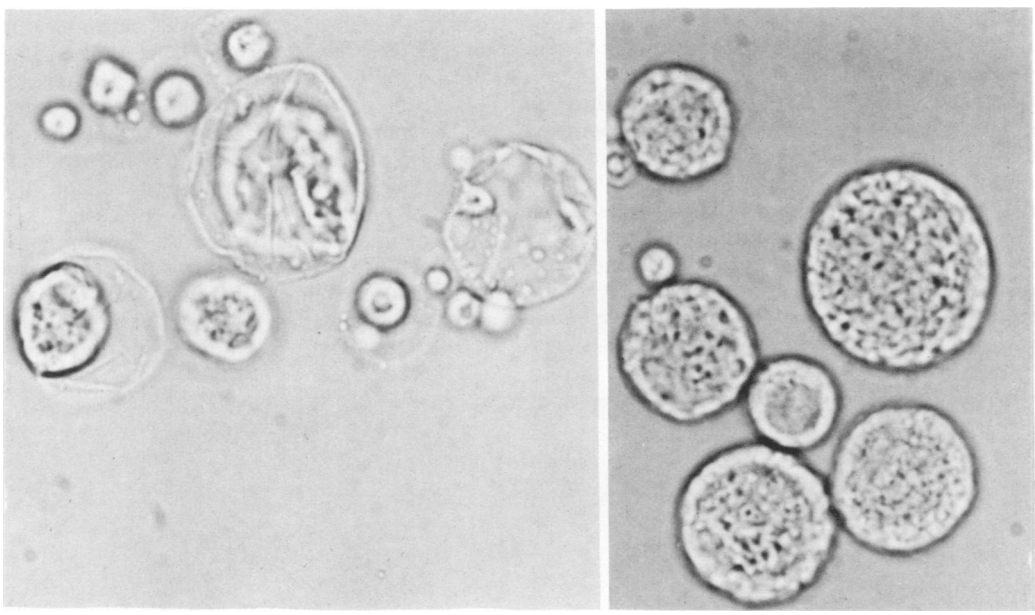

FIG. 2. *Immature spherules treated with lysozyme. (Left) Twenty micrograms of hen egg-white lysozyme per ml of distilled water (×1,000); (right) distilled water only (×1,000).*

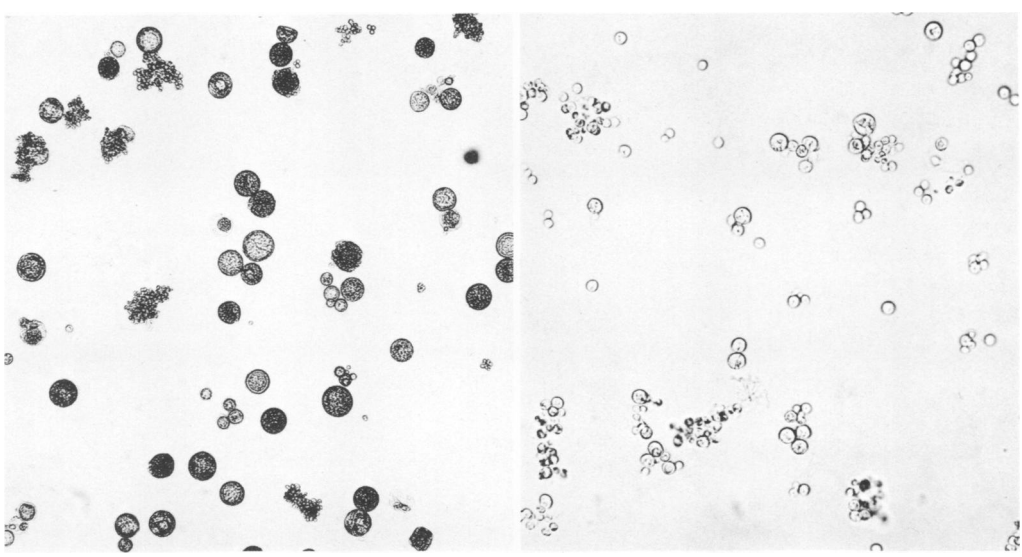

FIG. 3. *Untreated spherules grown in MCM-T. Left, 42 h (×100); right, 6 days (×250).*

rel shaped arthrospores were formed (Fig. 5, right). At equivalent concentrations, HEW lysozyme induced morphological changes similar to those induced by human lysozyme. At 50 and 100 μg of HEW lysozyme per ml the inoculum was associated with large amounts of amorphous lysozyme precipitate (Fig. 6). Newly synthesized hyphae devoid of precipitate were seen after 4 days of incubation growing away from the aggregation of inoculum and lysozyme precipitate. At concentrations of 20 μg/ml or less, HEW and human lysozyme did not yield visible precipitate.

Effects of chitinase and protamine on spherule growth. The effect of incorporating chitinase into MCM-T was limited to the rapid solubilization of the wall of fully mature spherules leaving only clusters of endospores. After 42 h of incubation in medium containing 70 μg of chitinase per ml nearly all mature endosporulating spherules were absent, replaced instead by endospore masses devoid of spherule wall, whereas in chitinase-free MCM-T less than 1% of the mature spherules were devoid of walls.

That the adverse effects of lysozyme on spherule growth might be due to the basic charge of lysozyme was shown by culturing spherules in the presence of protamine. This basic protein markedly inhibited growth: after 6 days of incubation absorbances at 450 nm of cultures containing 5, 10, or 20 μg of protamine per ml of MCM-T were only 1.6, 0.5, and 0.5%, respectively, the absorbance of the protamine-free control. The few spherules developing in flasks containing 5 μg of protamine per ml were similar in morphology to those developing in flasks containing 5 μg of lysozyme per ml. However, unlike lysozyme no concentration of protamine employed prompted reversion to the mycelial phase. Protamine at 10 μg/ml (2.3×10^{-6} M) or more permitted no development of the inoculum for 6 days, indicating that protamine was more inhibitory than lysozyme which at least permitted mycelial growth at 10 μg/ml (6.6×10^{-7} M). Spermine, spermidine, and

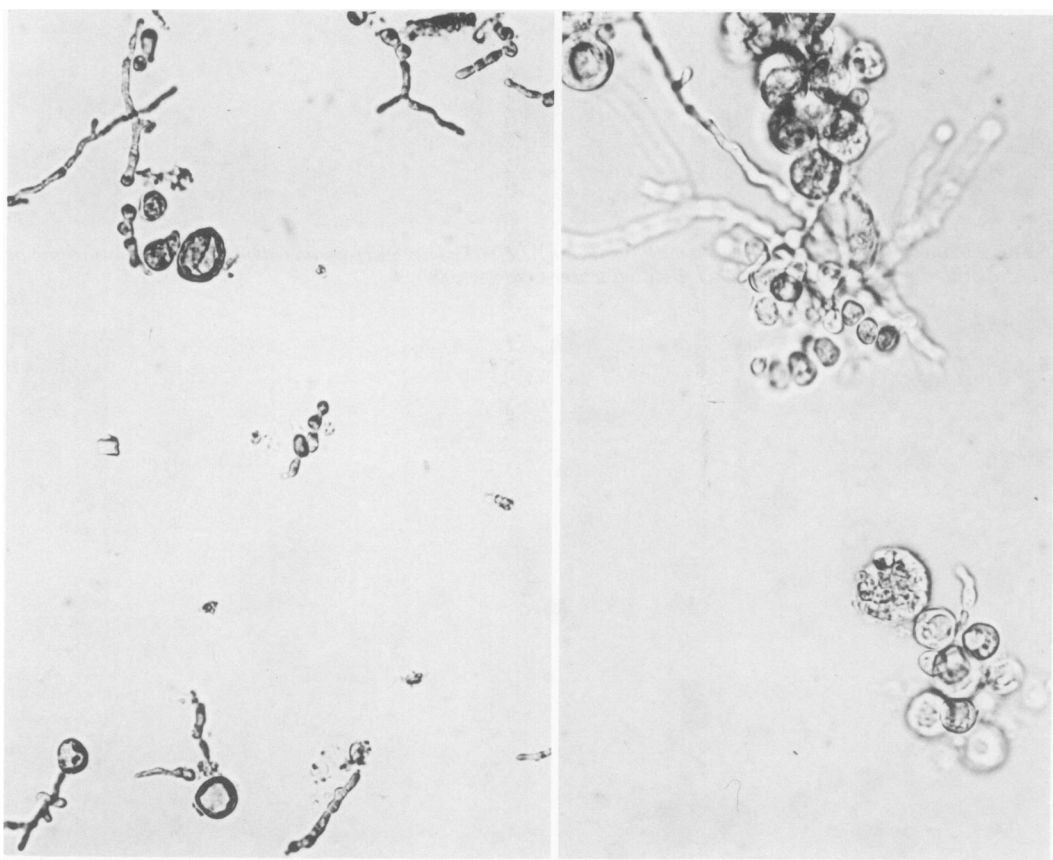

FIG. 4. *C. immitis cultured in MCM-T containing 5 μg of human lysozyme per ml. Left, 42 h (×450); right, 4 days (×450).*

putrescine, basic but not polymeric compounds, had no effect on normal spherule growth at 10^{-5}, 10^{-4}, or 10^{-3} M.

DISCUSSION

The interaction of lysozyme with spherule cytoplasmic membrane phospholipids seems to explain our results best. The formation of aqueous channels in the membrane resulting from lysozyme-membrane interaction would allow the egress of K^+ and nucleotides. The loss of these materials would eventually impair cell viability, especially of cells suspended in water. The ease with which lysozyme was eluted from

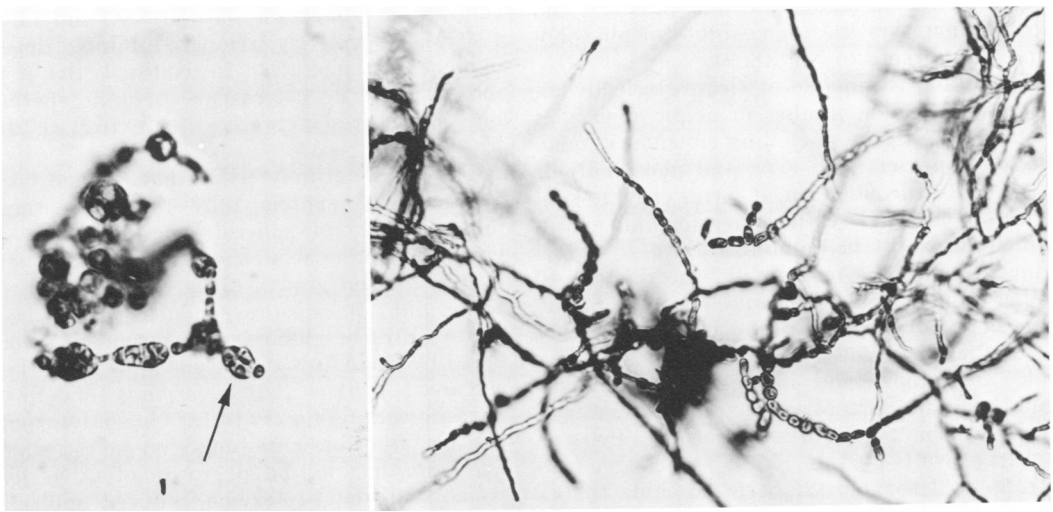

FIG. 5. *(Left) C. immitis cultures in MCM-T containing 10 μg of human lysozyme per ml (4 days). Note cell cleaving into endospores (×720). (Right) Hyphal growth developing in MCM-T containing 20 μg of human lysozyme per ml (6 days) (×325).*

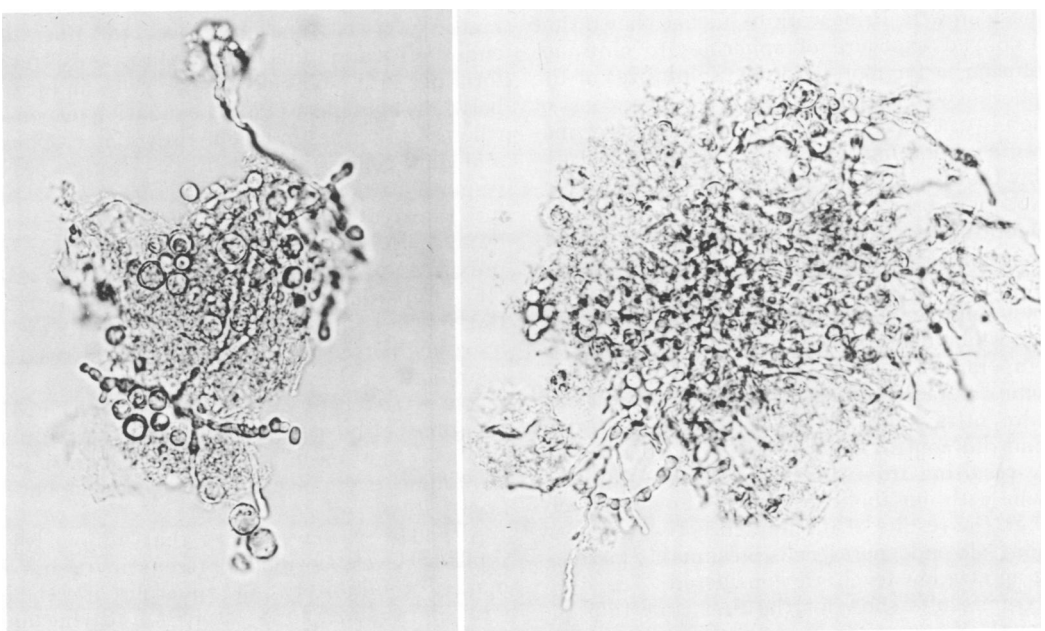

FIG. 6. *Precipitate associated with C. immitis interaction with lysozyme 4 days after inoculation. (Left) Fifty micrograms of hen egg-white lysozyme per ml of MCM-T (×250); (right) 100 μg of hen egg-white lysozyme per ml of MCM-T (×250).*

formalin-killed spherules by washing with cations argues in favor of cell surface lysozyme binding sites. Support for our contention that lysozyme inhibition or killing results from binding to the cytoplasmic membrane includes the demonstration that lysozyme electrostatically binds to artificial protein-phospholipid membranes (14), that it competes with Ca^{2+} for binding sites on acidic phospholipids (7), and that it increases the permeability of phospholipid membranes to Na^{+} (8, 9).

Lysozyme is capable of electrostatically interacting with a number of acidic molecules found in spherules including ribonucleic acid, deoxyribonucleic acid (17), and phospholipids (7, 8, 9, 14). In addition, it forms enzyme-substrate complexes with spherule wall chitin which leads to the solubilization of *N*-acetyl glucosamine (3). Accordingly, the damage to spherules by lysozyme could result from the interaction of the basic protein with a variety of structures including the spherule wall, cytoplasmic membrane, and intracellular organelles composed in part of molecules possessing a negative charge at intracellular pH. Because spherule walls contain chitin (19, 21), a substrate for lysozyme (2), it is probable that a fraction of the bound lysozyme was present in an enzyme-substrate complex. However, lysozyme interaction with spherule wall chitin is likely not responsible for the leakage of cytoplasmic materials or inhibition of spherule phase growth. Prior work by us has shown that prolonged exposure of spherules to chitinase solution, a far more effective solubilizer of the spherule wall than lysozyme (3), led to no loss of viability. Under the same conditions lysozyme-treated spherules were killed (3). It is conceivable that lysozyme damage results from interaction of lysozyme with biosynthetic or energy-generating mechanisms. However, for this interaction to occur the highly charged lysozyme molecule would have to be transported across the cytoplasmic membrane by a transport system which operates efficiently at 4 C, for at this temperature, as well as at 25 and 37 C, lysozyme-treated spherules leaked four to five times more ultraviolet 260-nm absorbing materials than did control spherules. Altered permeability resulting from direct interference by lysozyme with metabolic activities seems not likely when the results of its marked activity against 8-day-old endospores, cells presumably metabolizing at a low level, are considered.

Our results show for the first time that the growth of the spherule phase of *C. immitis* in vitro is markedly inhibited by concentrations of human lysozyme found in human serum (12, 13, 16). In part, inhibition of spherule growth appears to be due to the basic charge of lysozyme, for the basic protein protamine also inhibited spherule growth. However, in addition to basic charge, it seems essential that the inhibitory molecule be of a certain minimum size. Lysozyme (molecular weight 14,700) and protamine (molecular weight 4,300) at a concentration of 10 μg/ml (6.6×10^{-7} M and 2.0×10^{-6} M, respectively) strongly inhibited development of spherules. In contrast the low-molecular-weight polycations spermine, spermidine and putrescine were without effect at 10^{-3} M.

The basic nature of HEW and human lysozyme cannot explain fully the effect these proteins have of spherule morphogenesis in vitro. Protamine at a concentration which permitted limited growth (5 μg/ml), unlike lysozyme, did not cause reversion to the mycelial phase. Bizarre changes in morphology were noted by Gadebush and Johnson (4) when they cultured *Cryptococcus neoformans* in defined medium containing 250 to 1,000 μg of lysozyme per ml. Other basic proteins they investigated, like lysozyme, inhibited growth of *C. neoformans*, but only lysozyme induced multiple budding, the formation of pseudohyphae, and alterations in normal cell shape.

C. immitis induces a marked cellular infiltration into the infected tissues of the host. The nature of the infiltration is influenced by the maturity of the spherule. In general, liberated endospores and infectious arthrospores transforming into spherule phase cells induce a polymorphonuclear (PMN) exudative response, whereas spherules induce mainly a macrophage and giant cell response (1, 15, 18). Both types of response can release large amounts of lysozyme into the area surrounding *C. immitis* cells. Both human PMNs and macrophages contain approximately 7 to 8 μg of lysozyme per 10^6 cells (16). Supernatants of 6- to 24-h-old human PMN-rich exudates contain 34 to 112 μg of lysozyme per ml and if the cells are allowed to disintegrate, the lysozyme concentration can reach 400 to 900 μg/ml (16). Human PMN lysosomes also contain a heterogeneous population of other basic proteins (20), which like protamine may have an adverse effect on *C. immitis*.

Although we have shown that human lysozyme at levels normally present in tissues is markedly inhibitory for spherule growth in vitro, it remains to be seen if lysozyme and other basic proteins play a significant role in the humoral defense mechanism of the infected host.

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