Effects of Lysozyme and Chitinase on the Spherules of Coccidioides immitis In Vitro

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Spherules of Coccidioides immitis strain Silveira produced in vitro were treated with chitinase and lysozyme. The walls of merthiolate-killed mature endosporulating spherules were degraded by chitinase (500 μg/ml) and by lysozyme (100 and 500 μg/ml). Thus, as was visible through the light microscope, the spherule wall was reduced in thickness from 1 to 2 μm to less than 0.5 μm. The degradation was evident also by release of N-acetylglucosamine, three times as much N-acetylglucosamine being released by chitinase in 12 h as was released by lysozyme in 3 days. However, the effect of lysozyme on living mature spherules was in marked contrast to the effect of chitinase in that treatment with lysozyme led to marked reduction in viability. Exposure to lysozyme (500 μg/ml) for 48 h permitted survival of only 0 to 0.2% of spherules. Thinning of the walls was observed only in the larger spherules (25-35 μm) treated with lysozyme. By contrast, chitinase (500 μg/ml) led to complete dissolution of the walls of living mature spherules but the viability of the liberated endospores was unaffected during contact with chitinase for 48 h. Living non-endosporulating immature spherules and free endospores were also rendered nonviable by lysozyme but not by chitinase.

The development by Converse (4) of a medium for the propagation of the spherule form of Coccidioides immitis in vitro led to development of a killed spherule vaccine by Levine et al (12). This vaccine prepared by killing spherules with formaldehyde provided substantial protection in mice and monkeys subjected to inhalatory challenge with C. immitis (12, 13). It was subsequently tested in humans (14, 17) and was well tolerated at doses not greater than 5 mg, but only irregularly sensitized to coccidioidin. It was felt that increasing the antigenic mass injected (i.e., greater than that present in 5 mg of whole spherules) might lead to more consistent conversion of vaccinated individuals to coccidioidin sensitivity. This might be accomplished by solubilization of the antigen in the spherule wall which contains the immunogenic component(s) (11).

The present report deals with some studies in which enzymes were utilized in an effort to solubilize the spherule wall. Killed as well as viable spherules were tested. Chitin, a polymer of β 1-4 linked N-acetylglucosamine (NAG), has been shown to be present in the wall of spherules in vivo by histochemical methods (3, 24) and in the wall of cultured spherules by chemical methods. The effect of chitinase, and of lysozyme (muramidase) which is also known to hydrolyze chitin (3) were given particular attention.

MATERIALS AND METHODS

Preparation 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, Cobb and Smith (12). Immature spherules approximately 8 to 12 μm in diameter and without endospores were harvested at 24 h. Maturation of spherules occurred after 42 h of incubation. These were 30 to 35 μm in diameter and could contain up to several hundred endospores each representing a colony-forming unit (CFU). Spherules were harvested at that time. Merthiolate was added to the cultures to a final concentration of 0.01% (wt/vol), and the spherules were stored at 4°C for a minimum of 2 weeks until sufficient material had accumulated for analysis. After this exposure to Merthiolate no viable spherules or endospores were detected by plating on glucose yeast extract agar. Prior to enzyme treatment the spherules were washed several times with 10 mM phosphate buffer (pH 7.0) and suspended in the same buffer at a concentration of 13.01 mg (dry wt) per ml.

Treatment of spherules with lysozyme or chitinase. Lysozyme or chitinase (500 μg per ml) were
dissolved in distilled water containing 50 units per ml of crystalline penicillin G and 50 μg per ml of streptomycin to prevent bacterial contamination. The solution was sterilized by filtration through a membrane filter (Millipore Corp.). To 3.0 ml of the lysozyme solution was added 0.2 ml of the spherule suspension in phosphate buffer, giving a final phosphate concentration of 0.6 mM. The chitinase was made up in 10 mM phosphate buffer at pH 5.6. The enzyme-spherule mixture was placed in screw-cap test tubes (15 by 50 mm) and incubated at room temperature (23 ± 1 C) for varying times. Agitation was provided by a rotating tube drum operating at 60 rpm.

Enzymes. Hen egg-white lysozyme (lot 70 C-8110), lipase from wheat germ (lot 80 B-8120) and phospholipase-C (lot 120 C-6880) were obtained from Sigma (St. Louis, Mo.). Chitinase (lot 52926) and Pronase (lot 801930) were obtained from Calbiochem (San Diego, Calif.). Phospholipase and Pronase were dissolved (500 μg/ml) in 10 mM phosphate buffer at pH 8.0. Lipase was dissolved (500 μg/ml) at pH 7.0. The addition of lysozyme dissolved in distilled water to sterile MCM at final concentrations of 100 and 500 μg per ml resulted in the immediate precipitation of the enzyme, apparently a result of the ionic strength of the medium. However, lysozyme remained soluble in 10 mM phosphate buffer at pH 7.0.

Effect of lysozyme on Merthiolate-preserved spherules. After 3 days of exposure to lysozyme the spherules were recovered by centrifugation at 700 × g, washed once with 10 mM phosphate, pH 7.0, and stained with lactophenol cotton blue. The supernate was removed with a Pasteur pipette and was assayed for N-acetylhexosamine by the P-dimethylamino-benzaldehyde method (18).

Enzymatic treatment of viable spherules and endospores. Spherules were harvested from MCM by centrifugation at 700 × g followed by washing several times and suspending in 10 mM phosphate buffer, pH 7.0. Lysozyme in buffer was added to the washed spherules at concentrations of 0, 100, and 500 μg/ml. Chitinase in 10 mM phosphate buffer, pH 7.0, was similarly added to duplicate spherule suspensions to give a final concentration of 500 μg/ml. These mixtures were contained in a final volume of 4.0 ml in screw-cap tubes (15 by 150 mm).

Each tube contained 2.5 × 10^5 CFU per ml. CFU is used because the presence of spherules at varying stages of maturation and with variable numbers of endospores would preclude an accurate viable cell count. The tubes were incubated at room temperature (23 C) in a rotating tube drum at 60 rpm. After exposure to the chitinase for 3 days one of the duplicate suspensions was centrifuged at 700 × g, and the spherules were washed several times with buffer and suspended in lysozyme (500 μg/ml) buffer solution. At daily intervals a replicate loopful (approximately 0.01 ml) from each suspension was streaked over the surface of Sabouraud glucose agar (SDA) to estimate the surviving CFU. Plates were incubated at 34 to 35 C for at least 7 days.

In one experiment the viability of cells was estimated by the method of exclusion of buffered methylene blue (7), nonviable cells being stained blue. The numbers of spherules were estimated by hemocytometer count. Accompanying plate counts were carried out by streaking replicate loopfuls (approximately 0.01 ml) of spherule suspensions on SDA.

Liberated endospores were used in one part of these studies. Mature 42-h spherules were harvested as described above, washed, and suspended in 10 mM phosphate buffer pH 7.0. These were incubated for 7 days at 23 C. This led to release of endospores 2 to 3 μm in diameter. The majority of these were single endospores, and 80% of the CFU contained five or fewer endospores. The liberated endospores were washed several times and suspended in 4.0 ml of buffer containing 0, 100, or 500 μg of lysozyme per ml. Each tube contained 2.5 × 10^5 CFU per ml initially. After 5, 24, and 48 h exposure to lysozyme at 23 C, the viable CFU were estimated by streaking a loopful (approximately 0.01 ml) suspension over the surface of SDA.

RESULTS

Degradation of the spherule wall of killed spherules. Mature but unruptured endospore-containing spherules harvested at 42 h possess walls characteristically about 1.5 μm thick (Fig. 1A), and endospores 2 to 3 μm in diameter. A spontaneous thinning (autolysis?) of the wall of some mature endosporulating spherules occurred near the time of rupture and liberation of endospores (upper spherule in Fig. 1A). When these mature spherules killed with Merthiolate were treated with lysozyme (500 μg/ml) for 3 days, there was marked thinning of the double wall of all such spherules (Fig. 1B).

Treatment of killed mature spherules for 16 h with chitinase led to attenuation of the walls identical in appearance (Fig. 2A) to the thinning brought about by lysozyme after 3 days. Control spherules in 10 mM phosphate buffer at pH 5.6 were unaltered (cf. Fig. 1A). The walls of killed immature spherules that have yet to form endospores (<24 to 36 h) appear unaffected by either enzyme.

The action of lysozyme and chitinase was reflected by liberation of NAG. Treatment with lysozyme lead to an increase from 32.2 to 58.2 μg of NAG solubilized per mg of spherules, an increase of 80%. Chitinase liberated 122.3 μg of NAG per mg of spherules, an increase of 600% above the base-line of free NAG.

Attempts to digest further with additional enzymes the thin wall of spherules remaining after treatment with lysozyme or chitinase were unsuccessful. Thus, Pronase, wheat germ lipase, and phospholipase-C gave no change detectable with the light microscope (×970).

Effect of lysozyme and chitinase on viable spherules. Immature and non-endosporulating spherules harvested at 24 h and mature endo-
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sporulating spherules harvested at 42 h were treated with lysozyme and chitinase.

As shown in Table 1, spherules collected from MCM at 24 h were rendered nonviable by lysozyme at both concentrations tested, 100 and 500 µg per ml. This effect was evident in 24 h at which time there had been a reduction in CFU to less than 0.5% of the original inoculum of 2.5 x 10⁵ CFU per ml. Following 48 and 72 h exposure to lysozyme no growth was detected.

In contrast to the effect of lysozyme, incubation for up to 6 days in phosphate buffer or in chitinase solution (500 µg per ml) did not affect the viability of the 24-h spherules. Indeed, preincubation of the spherules in chitinase solution appeared to provide a measure of protection against subsequent treatment with lysozyme. Thus, treatment with lysozyme (500 µg per ml) only reduced the CFU to 32% at 24 h and at 72 h 0.1% of the original inoculum was still viable. One additional observation suggested that the chitinase might serve in a protective manner perhaps as a nutritional substrate: after incubation for 4 days at 37 C, the colonies from chitinase-treated spherules became visible sooner and averaged 4.0 mm in diameter compared with 0.5 mm for colonies of spherules exposed only to phosphate buffer or surviving after lysozyme treatment.

The morphology of lysozyme-treated 24-h spherules was altered. They appeared shrunken, and the cytoplasm stained only faintly with lactophenol-cotton blue. Twenty-four-hour spherules incubated for 6 days in chitinase or buffer maintained unaltered morphology. Spherules pretreated for 3 days with chitinase then with lysozyme for 3 days maintained the original morphology seen when harvested from MCM.

Treatment of viable, mature endosporulating 42-h spherules with lysozyme also reduced their viability markedly. By visual estimate, using the methylene blue exclusion test, fewer than 1% of spherules and endospores remained viable after 48 h of exposure to lysozyme (500 µg per ml). By plate count, 0.2% remained viable. Viability appeared only slightly affected by incubation of the mature spherules in phosphate buffer or chitinase (500 µg per ml).

The morphology of mature 42-h spherules exposed to lysozyme remained unchanged from their original appearance despite the marked

Fig. 1. A, Merthiolate-killed 42-h spherule exposed to phosphate buffer only for 3 days (x1,000). The wall of the upper spherule has undergone thinning spontaneously prior to the addition of merthiolate. Thinning of the wall precedes release of endospores. B, Merthiolate-killed 42-h spherule exposed to 500 µg/ml of lysozyme for 3 days.
FIG. 2. A, Merthiolate-killed 42-h spherules exposed to 500 μg of chitinase per ml for 16 h. B, Honeycomb-like matrix remaining after disruption of Merthiolate-killed 42-h spherule by sonic treatment.

TABLE 1. Viability of immature spherules of C. immitis following treatment with lysozyme or chitinase

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Days exposure</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>CFU/ml survival (%)</td>
</tr>
<tr>
<td>0.01 M phosphate buffer only</td>
<td>2.5 × 10^6</td>
</tr>
<tr>
<td>pH 7.0</td>
<td>100</td>
</tr>
<tr>
<td>Lysozyme (100 μg/ml)</td>
<td>1.0 × 10^2</td>
</tr>
<tr>
<td>Lysozyme (500 μg/ml)</td>
<td>3.0 × 10^2</td>
</tr>
<tr>
<td>0.1</td>
<td>N.G.</td>
</tr>
<tr>
<td>Chitinase (500 μg/ml)</td>
<td>2.5 × 10^4</td>
</tr>
<tr>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Chitinase (500 μg/ml)</td>
<td>2.5 × 10^6</td>
</tr>
<tr>
<td>3d, spherules washed and resuspended in lysozyme (500 μg/ml)</td>
<td>2.5 × 10^6</td>
</tr>
<tr>
<td>3d</td>
<td>100</td>
</tr>
</tbody>
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* CFU ± ½ log10.
* Original inoculum: 2.5 × 10^6 immature cells/ml.
* Not done.
* No growth.
* One colony detected, appeared grossly to resemble C. immitis.

decrease in viability. By contrast, treatment with chitinase left no clearly intact spherule walls and the clusters of endospores were very small. There were many solitary endospores compared with those incubated in plain buffer. The clusters of endospores remaining adherent after dissolution or partial dissolution of the spherule wall by incubation in plain buffer resemble those shown in Fig. 1B and 2A. Figure 2B is a phase-contrast photomicrograph of a
Merthiolate-killed mature spherule disrupted by exposure for 30 min to a Branson Sonifier (Melville, N.Y.) at 68 W using the 5.5-inch (about 14 cm) microtip. During disruption the spherule preparation was immersed in an ice bath. The honeycomb-like matrix in which endospores are enmeshed in the intact spherule is clearly visible. Chitinase appears to disrupt or dissolve this matrix at least in part.

**Effect of lysozyme on viable “starved” endospores.** In the preceding experiments viable spherules were studied shortly after being harvested from MCM at which time they were presumably metabolically active. Vital staining with methylene blue revealed that greater than 95% were viable. To study the effect of lysozyme on a uniform population of starved cells, endospores liberated from spherules maintained in phosphate buffer for 7 days were exposed to lysozyme. Lysozyme was again employed at concentrations of 100 and 500 μg per ml. One set of suspensions was incubated with added 1% glucose the other set without added glucose. Microscopically no difference was noted in the degree of aggregation of endospores incubated in the presence or absence of lysozyme. However, inasmuch as the endospores were in irregular clusters, viability was estimated qualitatively. There was no apparent decline in viability of endospores exposed to buffer for 48 h or to 100 or 500 μg of lysozyme per ml for 24 h, subcultures yielding nearly confluent growth. However, after 48 h, endospores exposed to either concentration of lysozyme yielded only scattered colonies. The killing effect of lysozyme on endospores was essentially the same in the presence or absence of added 1% glucose.

**DISCUSSION**

The action of lysozyme, an enzyme widely distributed in tissues and secretions (5), on bacteria has been widely described in the literature. The effect of this enzyme on fungi has not been widely studied. An antifungal effect has been described by some authors. Kamaya (10) reported that lysozyme was fungicidal for several species of *Candida* including *C. albicans*. Lysozyme was found to interfere with reversion of helicase-prepared protoplasts of *C. utilis* to complete cells (16). Schmidt (21) showed that lysozyme inhibited hyphal growth and induced degeneration of germ tubes or the release of cytoplasmic contents from conidia of *Peronospora tabacina*. Several basic proteins including lysozyme have been shown to cause the release of ultraviolet-absorbing material from *C. utilis* with an accompanying decline in viability (26). Lysozyme binds to the hyphal growing tip of soil fungi (15) and leads to agglutination of living cells of some yeasts including *C. albicans* and *C. stellatoidea* (9). Whether the latter contribute to an antifungal activity of this enzyme is uncertain.

The present study adds the spherule form of *C. immitis* to the list of fungi on which lysozyme has a deleterious effect. This effect was manifested two ways: first, there was degradation of the wall of Merthiolate-killed spherules. This partial dissolution of the wall left intact a thin layer enclosing intact endospores. Second, the lysozyme led to loss of viability of immature spherules and mature spherules including endospores.

Lysozyme treatment of spherules led to release of NAG presumably by hydrolysis of chitin in the cell wall. That this was apparently not the reason for lethality of the lysozyme was indicated by the survival of viable spherules and endospores after treatment with chitinase which led to thinning of the spherule wall and to liberation of markedly greater amounts of NAG. Thus, chitinase solubilized three times as much NAG from mature spherules in 12 h as lysozyme did in 3 days. Berger and Weiser (2) had shown a similar difference in activity of chitinase from *Streptomyces* sp. and hen egg-white lysozyme. Thus, chitinase released 130 mg of NAG per ml in 2 h from a solubilized chitin containing 500 μg/ml, whereas lysozyme released only 30 μg of NAG per ml after 72 h.

Neither chitinase nor lysozyme led to complete dissolution of the spherule wall. The residual thin wall may be due in part to the presence of glucan which Wheat and co-workers have suggested may be an additional component of the spherule wall (25). Spontaneous thinning of the wall occurred in spherules maintained in phosphate buffer and appeared to be a prelude to release of endospores. This may have resulted from an endochitinase or lysozyme-like enzyme in view of the similar thinning as seen with the light microscope (cf. Figs. 1A, 1B, 2A).

Lysozyme, a small basic protein with a molecular weight of 15,000 has been shown to bind non-enzymatically with a variety of acidic polymers including ribonucleic acid, deoxyribonucleic acid, Type II pneumococcal polysaccharide, hyaluronic acid, and Vi antigen (23). Under appropriate conditions, lysozyme has been incorporated into artificial membranes composed of protein and phospholipid. This resulted in a reorientation of the hydrophobic chains of the phospholipid molecules (19). Bakri and Wolfe (1) have recently shown that lysozyme can bring about coagulation of bovine milk casein micelles by interacting with the carbohydrate moiety of K-casein, the stabilizing glycoprotein of the casein micelles.
The loss of fungal cytoplasmic contents due to lysozyme has been reported by several authors (10, 21, 26). The lethal effect of lysozyme on spherules and endospores may be a consequence of cell membrane damage resulting from nonenzymatic binding of the strongly cationic enzyme to acidic components of the membrane such as phospholipids. Such binding may cause damaging conformational changes in membrane structure leading to loss of viability.

The fungicidal effect of lysozyme on spherules produced in vitro is of uncertain significance. While levels of lysozyme employed in this study (100 and 500 µg/ml) are not normally present in tissues, lysozyme levels of 400 to 900 µg/ml are found in neutrophile leukocyte extracts in man using skin chamber methods (22). Normal serum levels of lysozyme are 14 to 18 µg per ml. In the rabbit, increased lysozyme levels, 49 to 112 times normal serum levels, were detected in subcutaneous exudates induced by injection of glycogen. The significance of lysozyme present in macrophages as well as in neutrophils in inhibition of C. immitis spherules in vivo is unknown.

These studies were carried out with spherules and endospores removed from their growth medium. Investigations are now in progress to develop a system which will allow soluble lysozyme to be tested against growing and endosporulating phases of spherules in vitro.

ACKNOWLEDGMENTS

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ADDENDUM IN PROOF

Since the submission of this manuscript, we have learned that H. H. Gadebusch and A. G. Johnson (1966, J. Infect. Dis. 116:551) showed that lysozyme led to marked distortion of cells of Cryptococcus neoformans and exhibited a fungal static activity.

LITERATURE CITED