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Cryptococcus neoformans

III. Inhibition of Phagocytosis

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Isolated nonhydrolyzed cryptococcal polysaccharide is a rather specific potent inhibitor of the phagocytosis of *Cryptococcus neoformans* by human leukocytes in vitro. When an encapsulated strain of *C. neoformans* was cultured in the nonencapsulated state, the rate of phagocytosis was three times greater than when the encapsulated form was used. Our theory that capsular material plays a role in the pathogenesis of cryptococcosis requires (i) that *C. neoformans* exist in soil in a nonencapsulated state and (ii) that human phagocytes be capable of killing the organisms.

In a previous publication from this laboratory (3), it was reported that seven nonencapsulated mutants of *Cryptococcus neoformans* were avirulent for mice. Six of the mutants eventually reverted to an encapsulated state and in so doing gained varying degrees of virulence.

We subsequently demonstrated (4) that human leukocytes phagocytize cells of *C. neoformans* in vitro. The average rate of phagocytosis for nonencapsulated and encapsulated strains of *C. neoformans* was 78 and 24%, respectively. When the nonencapsulated strains reverted to the encapsulated state, the rate of phagocytosis decreased.

These results suggested that the capsule of *C. neoformans* plays a role in the virulence of the organism and may be responsible for inhibiting phagocytosis. The purpose of this paper is to present the results of more thorough investigation of the latter observation.

MATERIALS AND METHODS

The procedures used for the isolation and maintenance of the nonencapsulated mutants of *Cryptococcus neoformans*, the origin and maintenance of the parent encapsulated strain CIA, and the techniques used for measuring phagocytosis have been reported elsewhere in detail (3, 4).

Cryptococcal capsular material was obtained from an encapsulated *C. neoformans* strain CIA, after 3 days of incubation on Sabouraud Dextrose Agar at 26 C. Twenty grams of cells (collected with a rubber policeman), 20 g of glass beads (size 110, Minnesota Mining and Manufacturing Co., St. Paul, Minn.), and 20 ml of distilled water were added to a water-cooled sonic oscillator, Raytheon model DF 101 (Raytheon Co., South Norwalk, Conn.), and this suspension was oscillated at full power for 20 min. This treatment, as observed in India ink preparations, resulted in the release of 60 to 80% of the capsular material without disrupting cells. Cells and glass beads were removed by centrifugation at 12,000 × *g* for 15 min. The supernatant fluid was made up to 80% ethyl alcohol and stored overnight at 4 C. A white precipitate was collected by centrifugation at 12,000 × *g* for 15 min. This material was suspended in distilled water, reprecipitated with 80% ethyl alcohol, resuspended in distilled water, and stored at −18 C. The precipitate was quantitated by determining the dry weight of various samples.

The organisms used in phagocytosis experiments, *Escherichia coli*, *Staphylococcus aureus*, *Saccharomyces cerevisiae*, *Candida albicans*, and *Candida* sp., were obtained from stock cultures in the Department of Microbiology, University of Oklahoma School of Medicine. Bacteria were cultured on BBL Blood Agar Base (Trypticase Soy Agar) at 37 C and incubated overnight. The yeast were cultured on Sabouraud Dextrose Agar at 26 C for 2 days. These organisms, suspended in human serum, were used in phagocytosis experiments at a final concentration of approximately 2×10^6 organisms/ml. In all instances, the suspending serum and the leukocytes were from the same donor.

Polysaccharides obtained from Y.-T. Li, starch arrowroot, guar gum, hyluronic acid, chondroitin sulfate, and gum acacia, were suspended in water and added to phagocytosis experiments to provide a final concentration of 400 μg/ml.

To test the effect of various carbohydrates and hydrogen ion concentrations on the size of the cell and capsule of *C. neoformans*, a modified Littman's medium (5) was prepared as follows: KH_2PO_4, 2 g; $(NH_4)_2SO_4$, 2 g; $MgCl_2 \cdot 6H_2O$, 0.2 g; $CaCl_2 \cdot 2H_2O$, 0.02 g; $FeCl_2 \cdot 4H_2O$, 0.04 g; $MnCl_2 \cdot 4H_2O$, 0.0015 g; $NaNO_3$, 0.0015 g; agar, 20 g; distilled water, 1,000 ml. One of the following carbohydrates, D-glucose, D-fructose, sucrose, D-mannose, sodium gluconate, or

maltose, was added to 1 liter of basal medium to give a final concentration of 1%. Six samples of these media were adjusted to a final *p*H of 5.0, 5.5, 6.0, 6.5, 7.0, and 7.5. After autoclaving, thiamine HCl, in a concentration of 0.001 g/liter of medium, was added. Plates were poured, and, after solidification, they were inoculated with *C. neoformans* strain CIA and incubated at 26 C for 2 to 3 days. Plates of Sabouraud Dextrose Agar, *p*H 5.7, were inoculated as a control.

The method used for measuring capsular thickness and cell size was described by Ishaq, Bulmer and Felton (Mycopathol. Mycol. Appl., *in press*).

RESULTS

The capsular constitutents of *C. neoformans* (2), galactose, glucuronic acid, mannose, and xylose, were detected, by paper chromatography, in the hydrolyzed capsular material of *C. neoformans* strain CIA.

To determine the effect of the capsular material on the phagocytosis of nonencapsulated mutants of *C. neoformans* by human leukocytes in vitro, varying amounts of capsular material were added to reaction mixtures consisting of nonencapsulated mutant, leukocytes, and autologous serum. Three different mutants, and blood from five different normal human donors, were used in these studies. The results are shown in Fig. 1. When no capsular material was added, the mean percentage of phagocytosis was 72. When as little as 177 μg of capsular material per ml was added, the value decreased to 28. As the amount of capsular material was increased, the rate of phagocytosis decreased further until maximal inhibition was noted at a level of approximately 1,000 μg/ml. With the encapsulated strain CIA, the rate of phagocytosis was 18%.

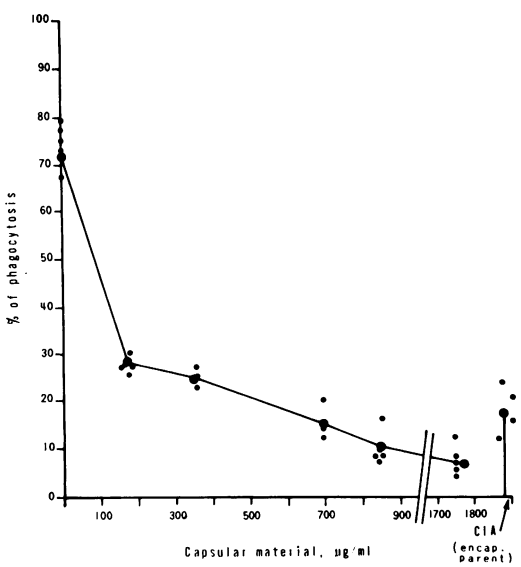

FIG. 1. *Inhibition of phagocytosis of nonencapsulated mutants of Cryptococcus neoformans by capsular material in vitro.*

No inhibition of phagocytosis was observed with capsular material that had been hydrolyzed for 4 hr in 1.5 N HCl at 100 C.

To determine whether the inhibition of phagocytosis by cryptococcal capsular material was specific for *C. neoformans*, 464 μg/ml of cryptococcal capsular material, from *E. coli*, *S. aureus*, *S. cerevisiae*, *Candida albicans*, and *Candida* sp., was used in phagocytosis experiments. The capsular material did not inhibit the uptake of any of these microorganisms by human leukocytes in vitro.

To test the specificity of inhibition of phagocytosis by the cryptococcal capsular material, heterologous polysaccharides were added to tubes containing human leukocytes, autologous serum, and cells of the M7 mutant strain of *C. neoformans*. As seen from the data presented in Table 1, phagocytosis was not depressed by any of these polysaccharides, with the possible exception of hyluronic acid.

Since our investigations thus far were concerned solely with nonencapsulated mutants and the encapsulated parent strain, it was decided to attempt to culture the parent strain in a nonencapsulated state. It will be noted (Table 2) that, when sucrose, dextrose, and fructose were used as carbon sources, no visible capsule was produced at *p*H 5.0. As the *p*H increased, a capsule was observed. When mannose or sodium gluconate was used as a carbon source, a capsule was observed at all *p*H values; however, the capsule was much smaller at the lower *p*H values. Cells of CIA grown in the presence of four different carbon sources (sucrose, dextrose, fructose, mannose) at *p*H 7.5 and 5.0 were used in phagocytosis experiments. The results are presented in Table 3. On Sabouraud Agar, at a *p*H of 6.7, the capsule thickness was 2.5 μ and

TABLE 1. *Effect of various polysaccharides on the phagocytosis of Cryptococcus neoformans M7, by human leukocytes in vitro*

Polysaccharide	Amt (μg/ml)	Phagocytosis (%)
None	0	88
Starch arrowroot	400	88
Guar gum	400	80
Hyaluronic acid	400	60
Chondroitin sulfate	400	80
Gum acacia	400	88
Cryptococcal	353	23

TABLE 2. *Measurements of Cryptococcus neoformans strain CIA grown on a modification ofLittman's medium containing various carbohydrates and Sabouraud Dextrose Agar*

Carbohydrate	pH	Diameter (μ)		
		Cell + capsule	Cell only	Capsule thickness[a]
Sucrose	5.0	4.5	4.5	0.0
	5.5	7.3	4.9	1.2
	6.0	7.0	5.0	1.0
	6.5	7.3	5.1	1.1
	7.0	7.0	5.0	1.0
	7.5	8.6	5.0	1.8
Dextrose	5.0	5.2	5.2	0.0
	5.5	6.9	5.3	0.8
	6.0	7.1	4.9	1.1
	6.5	7.9	4.7	1.6
	7.0	9.4	5.6	1.9
	7.5	10.3	4.1	3.1
Fructose	5.0	5.3	5.3	0.0
	5.5	5.3	5.3	0.0
	6.0	5.8	4.0	0.9
	6.5	6.8	4.4	1.2
	7.0	7.6	4.6	1.5
	7.5	9.8	4.6	2.6
Maltose	5.0	7.1	4.9	1.1
	5.5	6.7	4.7	1.0
	6.0	6.1	4.3	0.9
	6.5	6.9	4.9	1.0
	7.0	7.4	5.2	1.1
	7.5	7.6	4.0	1.8
Mannose	5.0	4.9	3.9	0.5
	5.5	4.8	3.6	0.6
	6.0	5.6	3.8	0.9
	6.5	8.2	5.6	1.3
	7.0	10.1	4.9	2.6
	7.5	10.3	3.7	3.3
Sodium gluconate	5.0	5.1	3.7	0.7
	5.5	5.6	4.0	0.8
	6.0	5.4	3.8	0.8
	6.5	6.0	4.0	1.0
	7.0	6.1	4.1	1.0
	7.5	10.7	6.9	1.9
Sabouraud Dextrose agar	6.7	10.4	5.4	2.5

[a] Capsule thickness was determined by subtracting the diameter of the cell from the diameter of the cell plus capsule, and dividing by two (C. M. Ishaq, G. S. Bulmer, and F. G. Felton, Mycopathol. Mycol. Appl., *in press*).

TABLE 3. *Phagocytosis and capsular thickness of Cryptococcus neoformans grown on a modification of Littman's capsular medium containing various carbohydrate sources*[a]

Substrate	pH	Capsule thickness (μ)	Phagocytosis[a] (%)
Sabouraud...	6.7	2.5	24
Sucrose.....	7.5	1.8	24
Sucrose.....	5.0	0.0	70
Dextrose.....	7.5	3.1	16
Dextrose.....	5.0	0.0	70
Fructose.....	7.5	2.6	28
Fructose.....	5.0	0.0	72
Mannose.....	7.5	3.3	20
Mannose.....	5.0	0.5	44

[a] In vitro, with human leukocytes.

the rate of phagocytosis was 24%. When sucrose, dextrose, and fructose were used as carbon sources, a capsule was present at *p*H 7.5, and the rate of phagocytosis ranged from 16 to 28%. However, with these same carbon sources, no capsule was present at *p*H 5.0, and the rate of phagocytosis ranged from 70 to 72%. When the carbon source was mannose, a large capsule was observed at *p*H 7.5, and the rate of phagocytosis was 20%, whereas at *p*H 5.0 a small capsule was present and the rate of phagocytosis was 44%. CIA cells cultured on sucrose as the carbon source, at *p*H 5.0, were incubated for 4 min at 37 C in a saline suspension containing 400 μg/ml of capsular material from CIA. These cells were then washed twice in saline, by centrifugation at 1,000 × *g* for 10 min, and used in phagocytosis experiments. The rate of phagocytosis in these CIA cells was 45% lower than for similar cells that were not preincubated with cryptococcal capsular material.

DISCUSSION

The addition of 177 μg of cryptococcal capsular material per ml to nonencapsulated mutants of *C. neoformans* resulted in a threefold suppression of phagocytosis of the organisms by human leukocytes in vitro. The rate of phagocytosis decreased further in the presence of increasing quantities of capsular material. Approximately 600 μg/ml was required to suppress phagocytosis to the level seen with the encapsulated parent strain. These results suggest that small amounts of crytococcal capsular material inhibit the uptake of nonencapsulated cells of *C. neoofrmans* by human leukocytes in vitro. The inhibition of phagocytosis by cryptococcal capsular material is rather specific, since the cryptococcal capsular material did not inhibit the uptake

of other yeasts and bacteria. Furthermore, hydrolyzed cryptococcal polysaccharide or other polysaccharides were ineffective in suppressing the phagocytosis of *C. neoformans*. Some information regarding the site of the inhibitory action was gained from experiments in which nonencapsulated cells of *C. neoformans* were preincubated with cryptococcal capsular material, washed, and then added to the phagocytic system. Since phagocytosis of these cells was depressed, it appeared that the specificity of the capsular material for *C. neoformans* resulted from its adherence to the cryptococcal cell.

When the encapsulated parent strain was cultured in the nonencapsulated state, the rate of phagocytosis was approximately three times greater than that seen in cells of the same strain cultured under conditions which induced capsule formation. Thus, the cryptococcal capsule appears to be a potent inhibitor of phagocytosis of *C. neoformans* cells by human leukocytes in vitro.

The fact that *C. neoformans* has always been considered to exist only in the encapsulated state makes the findings reported here of questionable significance. However, Ishaq, Bulmer, and Felton (Mycopathol. Mycol. Appl., *in press*) showed that when *C. neoformans* is cultured in soil its capsule becomes smaller, and it is possible that when the organism survives in soil and is inhaled by man it may be nonencapsulated. If this is true, then the finding that capsular material inhibits phagocytosis assumes increasing significance. In theory, the nonencapsulated infective particle would be phagocytized readily, and no disease would result. However, if the phagocytic process in the host were, for some reason, depressed, then the infective particle would not be so readily eliminated, and the lag phase would afford the nonencapsulated organism an adequate period of time to develop a capsule.

Although humoral and cellular factors were not investigated in the present studies, it should be emphasized that these factors may be involved in defense against *C. neoformans*. The results of studies with mice suggest that cellular factors are of utmost importance (1, 6). However, the mouse is a susceptible host, whereas crytptococcosis is rare in humans, despite the fact that the etiological agent has been isolated from soil throughout the world. In humans, defense against crytptococcosis may involve both cellular factors and serum antibody.

If *C. neoformans* exists in the soil in a nonencapsulated state, then nonencapsulated organisms should be used more widely in the study of cryptococcosis, e.g., in serological procedures and in studies on skin-testing antigens.

Our theory that the capsule of *C. neoformans* plays a role in the pathogenesis of cryptococcosis requires (i) that *C. neoformans* exist in soil in a nonencapsulated state and (ii) that human phagocytes be capable of killing the nonencapsulated organisms. These factors are currently being investigated in this laboratory.

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