

Cryptococcus neoformans

II. Phagocytosis by Human Leukocytes

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Twenty-four per cent of the leukocytes from healthy human subjects phagocytized an encapsulated strain of *Cryptococcus neoformans*. Phagocytosis was approximately three times more effective with nonencapsulated mutants of *C. neoformans*. When the mutants reverted to the encapsulated state, the percentages of phagocytosis decreased. These data indicate that cryptococcal polysaccharide inhibits the phagocytosis of *C. neoformans* by human leukocytes.

Giant cells containing *Cryptococcus neoformans* have been observed by histopathological examination of materials from cases of cryptococcosis in humans (4, 5, 8, 9). The observation that human leukocytes engulf yeast cells of several pathogenic fungi in vitro was reported by Wang and Schwarz (12).

Drouhet and Segretain (3) reported that cryptococcal polysaccharide inhibits the migration of guinea pig leukocytes. Gadebush (6, 7) demonstrated that a small capsule variant of *C. neoformans* was phagocytized more readily by leukocytes from anemic mice than was a large capsule variant. As a result of studies with large and small capsule strains, Louria and Kaminski (10) proposed that immunity to cryptococcosis in mice is related to "tissue bound" rather than circulating antibody. Abrahams (1) also suggested that in mice the protective mechanism against cryptococcosis exists primarily at the cellular level.

The isolation of seven nonencapsulated mutants of a human isolate of *C. neoformans* was reported previously (2a). The organisms were avirulent for mice. After repeated subculturing, six of the strains reverted to the encapsulated state and regained various degrees of virulence. Since these observations suggest that the capsule may be an important factor in the virulence of *C. neoformans*, it was decided to investigate possible differences between these strains and the parent encapsulated organism with regard to phagocytosis. This paper, and the ensuing one, report on the in vitro phagocytosis of *C. neoformans* by human leukocytes, the differences in percentage of phagocytosis between the encapsulated parent strain and seven nonencapsulated mutants, and the inhibition of phagocytosis by cryptococcal capsular material.

MATERIALS AND METHODS

Eight strains of *C. neoformans* were used in these studies: CIA, an encapsulated strain of human origin, and seven mutants, which, upon original isolation following ultraviolet light irradiation of CIA, were nonencapsulated and avirulent for mice. The isolation and maintenance procedures for these organisms were reported previously (2a).

Preliminary studies were performed to determine whether human leukocytes could phagocytize cells of *C. neoformans* in vitro. Single drops of whole blood from finger punctures were allowed to clot on a clean microscope cover slip. Within 30 min leukocytes adhered to the cover slip and the fibrin clot was removed. Cells of *C. neoformans* suspended in autologous serum were added to the leukocyte preparation. Visual observations were made with the aid of a phase-contrast microscope.

All subsequent phagocytosis experiments were conducted with freshly collected human blood. Healthy technicians, students, and faculty members of the University of Oklahoma School of Medicine served as donors. Serum used for the cryptococcal suspending medium was obtained from 20 ml of blood which was drawn aseptically by venipuncture and allowed to clot in sterile tubes.

The method of Braunsteiner et al. (2) was used for the isolation of 75 to 85% of the leukocytes in the blood samples. A 20-gauge disposable needle affixed to a 20-ml plastic syringe containing 100 units of heparin was used for venipuncture. A 10-ml amount of venous blood was drawn into the syringe and 4 ml of a 3.5% solution of polyvinylpyrrolidone (type NP-K30) in 0.9% NaCl was added immediately. The needle was removed from the syringe and replaced with another 20-gauge disposable needle. The syringe was rotated momentarily and placed at a 45° angle in a 500-ml beaker with the needle pointed upward. After 1 hr at 8 C the syringe was removed from the beaker, the needle was bent to a 45° angle, and the supernatant fluid was expelled carefully into a sili-cized glass tube (13 × 100 mm) by pressing slowly upward on the plunger of the syringe. The superna-

tant fluid contained the plasma and leukocytes, with little red blood cell contamination. The number of leukocytes in the suspension was determined by counting a sample in a standard hemocytometer. The final concentration of leukocytes ranged from 3,800 to 4,500 cells per mm³. About 95% of the leukocytes were viable as determined by the trypan blue test (11).

One ml of a 2×10^6 to 4×10^6 cryptococcal cell suspension in autologous serum and 1 ml of the leukocyte suspension were added to siliconized glass tubes (13 \times 100 mm). The tubes were placed vertically in an Eberbach Rotator in a 37 C incubator and shaken at 180 oscillations/min for 1 hr. A drop of the mixture from each tube was then placed on a microscope slide, air-dried, and stained with Wright's stain. The slides were examined microscopically with the aid of an oil immersion lens. One hundred leukocytes were counted, and the number of leukocytes containing cryptococcal cells was recorded as the percentage of phagocytosis. The phagocytic index was determined by counting the number of yeast cells ingested per 100 phagocytes.

RESULTS

The leukocytes in the finger puncture preparations appeared very active, with extending pseudopods and numerous rapidly moving cytoplasmic granules. Within a few minutes, it was readily apparent that the granulocytes had engulfed some of the yeast cells. The phagocytic process appeared to be no different from that described for other particulate matter. The entire process required less than 2 min after initial contact between the cell and phagocyte. It is possible, however, that the heat generated by the microscope lamp may have artificially increased the activity of the leukocytes. As far as could be determined, the neutrophil was the principal phagocytic cell.

When the nonencapsulated mutants, M1 through M7, were used in place of the encapsulated parent strain, active phagocytosis by neutrophils was observed (Fig. 1). Attempts to quantitate the differences in phagocytic activity between CIA and the nonencapsulated mutants were not successful. However, it was obvious that more mutants than encapsulated parent cells were engulfed per phagocyte.

The inability to obtain quantitative data and the limitations as to the types of experiments that could be conducted with the slide method prompted a change to the tube procedure.

Sera and leukocytes from 15 individuals were tested for phagocytic activity against the encapsulated strain CIA. An average of 24% of the leukocytes engulfed yeast cells (Table 1, column 3). Phagocytic indices ranged from 0.16 to 0.24.

The sera and leukocytes of 35 individuals were tested for phagocytic activity against cells of the

TABLE 1. *Phagocytosis of encapsulated Cryptococcus neoformans (CIA) and mutants (before and after reversion to encapsulated organisms)*

<i>C. neoformans</i> strain no.	Before reversion		After reversion ^a	
	Encap- sulated organisms	Phago- cytosis	Encap- sulated organisms	Phago- cytosis
	%	%	%	%
Mutant M1 ...	0	76	60	64
Mutant M2 ...	0	84	51	68
Mutant M3 ...	0	82	98	24
Mutant M4 ...	0	69	75	50
Mutant M5 ...	0	83	80	44
Mutant M6 ...	0	74	80	40
Mutant M7 ...	0	79	0	72
Parent CIA ...	100	24	100	26

^a After 12 months of weekly subculturing.

seven nonencapsulated mutants. The mean percentage of phagocytosis ranged from 69 to 84 (Table 1, column 3). The mean phagocytic index was 1.27.

As reported previously (2a), six of the seven mutants began to revert to the encapsulated state after 5 months of weekly subculturing. After 12 months of subculturing, a high percentage of the cells of M1 through M6 were encapsulated (Table 1, column 4). The cells of M7 remained nonencapsulated. As shown in Table 1, the percentage of phagocytosis for M1 through M6 after reversion to the encapsulated state ranged from 24 to 68, with a mean of 48. At this time, an average of 74% of the cells in these strains were encapsulated. In contrast, the average percentage of phagocytosis was 78 when none of the six strains had a capsule. Throughout the subculturing process, M7 remained nonencapsulated and the percentage of phagocytosis remained high i.e., 79% after original isolation and 72% after 12 months of subculturing. The percentage of phagocytosis for the encapsulated parent strain remained low, i.e., 24% originally and 26% after 12 months of subculturing.

DISCUSSION

In a series of preliminary experiments in which human leukocytes were incubated with a human isolate of encapsulated *C. neoformans* on a microscope slide and observed through a phase-contrast microscope, the actual engulfment of yeast cells was seen. Phagocytosis of nonencapsulated mutants of *C. neoformans* was also followed. Although the latter cells appeared to be engulfed more readily by leukocytes than were cells of the encapsulated strain, it was difficult to quantitate

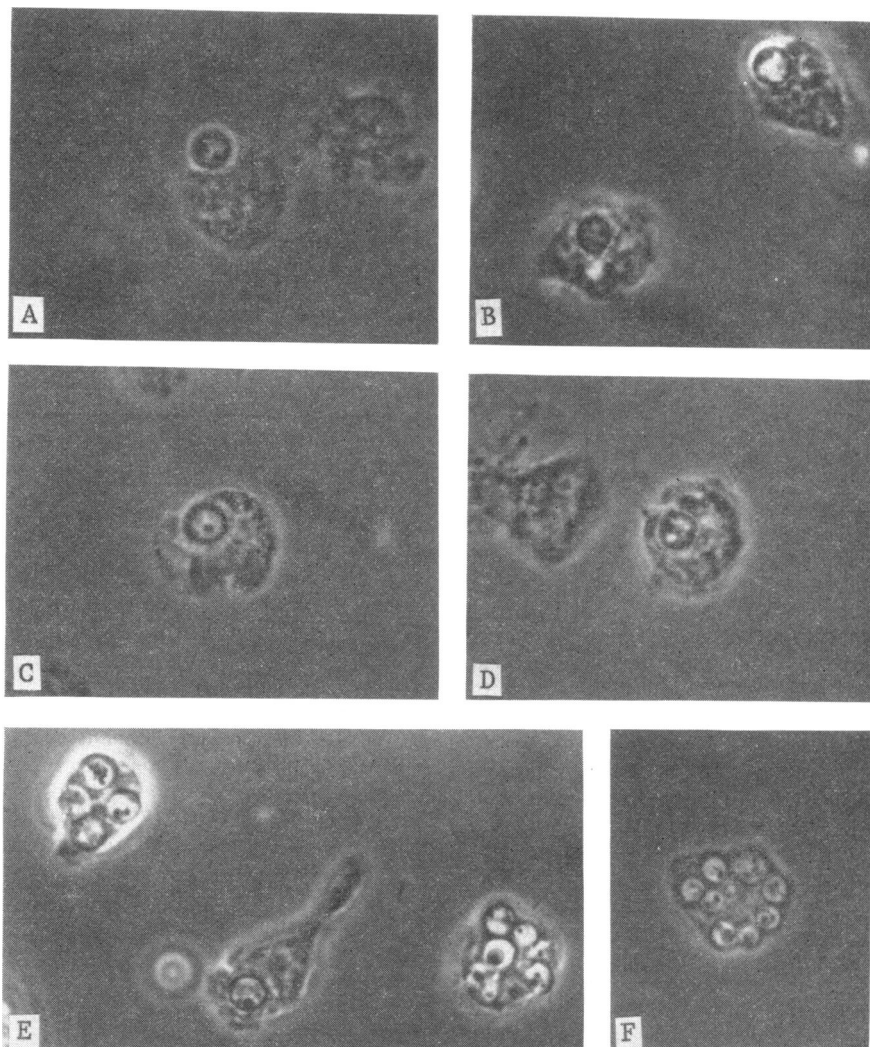


FIG. 1. Phagocytosis of nonencapsulated *Cryptococcus neoformans* (M7) by human leukocytes in vitro: (A, B, C, D) the phagocytic process; (E) two leukocytes containing numerous yeast cells and one phagocyte with extended pseudopod; (F) a leukocyte containing approximately nine yeast cells.

the differences by use of the slide technique. For this reason, and because the types of experiments that could be conducted with this procedure were limited, a tube technique was adapted for further investigation. With this technique, the average percentage of phagocytosis for the encapsulated cells of *C. neoformans* was 24. Phagocytosis was approximately three times as effective with the nonencapsulated cells. These results seemed to indicate that the cryptococcal capsule plays a role in the inhibition of phagocytosis by human leukocytes in vitro. This possibility gained some support as a result of further experimentation. After subculturing the nonencapsulated mutants

weekly for several months, six of the strains reverted, in varying degrees, to the encapsulated state. The results of phagocytosis experiments with these reverted mutants supported the contention that the capsule depresses phagocytosis.

Throughout the months of subculturing, the parent strain retained its capsule and the percentage of phagocytosis was consistently low. Conversely, one of the mutants remained nonencapsulated and the percentage of phagocytosis was consistently high.

These data suggest that the capsule of *C. neoformans* inhibits the phagocytosis of the organism. Quantitative data on the inhibition of phag-

ocytosis by cryptococcal capsular material will be presented in a subsequent article in this series (J. Bacteriol., *in press*).

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