INFECTION AND IMMUNITY, Apr. 1972, p. 491-498
Vol. 5, No. 4
Printed in U.S.A.
Copyright © 1972 American Society for Microbiology

In Vitro Phagocytosis and Intracellular Fate of Variously Encapsulated Strains of *Cryptococcus neoformans*

THOMAS G. MITCHELL AND LORRAINE FRIEDMAN

Department of Microbiology and Immunology, Tulane University School of Medicine, New Orleans, Louisiana 70112

Received for publication 8 November 1971

Five isolates of *Cryptococcus neoformans* type A with stable capsular thicknesses were used. Three of the isolates had capsules of medium size, one had a minimal capsule, and the other, a large capsule. Peritoneal exudate cells from Lewis rats were cultured on cover slips in Leighton tubes containing medium 199 and 20% fresh, isologous normal rat serum. Yeast cells were added to the Leighton tube cultures, and, 2 hr later, the extracellular yeasts were rinsed out. Cover slips were removed from some tubes for Wright staining and measurement of both phagocytosis and loss of macrophages. The remaining tubes were reincubated and sampled at 24 or 48 hr. To determine fate of yeast cells after ingestion, washed cover slips were inverted onto agar slide cultures, and specific macrophages were observed in situ for subsequent multiplication of their intracellular yeasts. More than half of the macrophages survived 24 to 48 hr of exposure to different strains of *C. neoformans*, with small, medium, or large capsules. Phagocytic activity was dependent upon a heat-labile factor in normal rat serum. The number of yeast ingested by macrophages was inversely proportional to the capsular size. Although most of the ingested yeasts were resistant to intracellular killing, the agar culture technique clearly demonstrated that many were unable to multiply, presumably dead. Three of the isolates were more susceptible than the other two, and the fate of these yeasts after engulfment was not correlated with their capsular size.

Cryptococcus neoformans (Sanfelice) Vuillemin, 1901, the etiological agent of cryptococcosis, is a ubiquitous, polysaccharide-encapsulated yeast. Human infection is believed to result from inhalation. Since *C. neoformans* can be isolated with facility from the environment but the incidence of cryptococcal disease is relatively low, it is assumed that many more individuals inhale the yeasts than actually become diseased.

Several observations point to a defensive role for the reticuloendothelial tissue in normal host defense against cryptococcosis: (i) granulomata are often found in the infected lungs (1, 11); (ii) specific antibodies to *C. neoformans* are not protective against either natural (9, 11) or experimental (10, 12, 13) infection; and (iii) many of those who become diseased have impaired reticuloendothelial tissues (14, 20).

The cryptococcus-macrophage interaction would seem to be the fundamental level at which to investigate the mechanism of pathogenesis in, and defense against, cryptococcosis. Under experimental conditions, phagocytosis of cryptococci by normal host leukocytes has been shown to occur (4-8, 17, 18), but the factors and the kinetics involved have not been described in detail. Evidence indicates that the polysaccharide capsule inhibits phagocytosis (4, 5, 7), but there is no information on the effect, if any, of capsular material on macrophages, nor whether the capsule protects the yeasts against intracellular digestion. In fact, whatever deleterious effect exposure to cryptococci may have on normal host reticuloendothelial cells (8) has not been studied in detail. Neither has anyone demonstrated, by a direct method, the fate of individual cryptococci after engulfment.

One purpose of this study was to develop a standard system for the in vitro phagocytosis of *C. neoformans* by macrophages from a host comparable to normal humans in cryptococcal resistance, which would permit quantitative assays of (i) extent of phagocytosis, (ii) effect of cryptococci on macrophages, and (iii) fate of the ingested cryptococci. Thereafter, different strains of *C. neoformans*, which varied in the

respective sizes of their capsules, were evaluated to investigate the possible role of capsular material in the interaction between yeasts and host cells.

MATERIALS AND METHODS

Cryptococcal isolates. The strains of *C. neoformans* used (designated 6L, 15S, 98M, 110M and 145M) were originally isolated several years ago from individual cases of cryptococcosis and maintained on Sabouraud dextrose slants at 37 C with fortnightly transfer. Serotyping by Morris Gordon (New York State Dept. of Health, Albany) showed them all to be type A. To measure capsular size, suspensions of the yeasts were examined with India ink negative staining. Strain 6L was highly encapsulated (average width of capsule, 3.5 μm), strain 15S possessed minimal capsular material (0.6 μm), and the other three had capsules of medium size (1.0–3.0 μm; Fig. 1).

Phagocytosis. For each experiment, two to five female rats (inbred, Lewis strain, 250 ± 25 g) were stimulated by intraperitoneal injection of 20 ml of oyster glycogen (1%, w/v) in Hanks balanced salt solution (HBSS). Three days later, they were exsanguinated by cardiac puncture, the serum was recovered, and peritoneal exudate cells were aseptically harvested by rinsing the incised cavities with 50 ml of cold HBSS containing 0.5 unit of heparin per ml and antibiotics (100 units of penicillin per

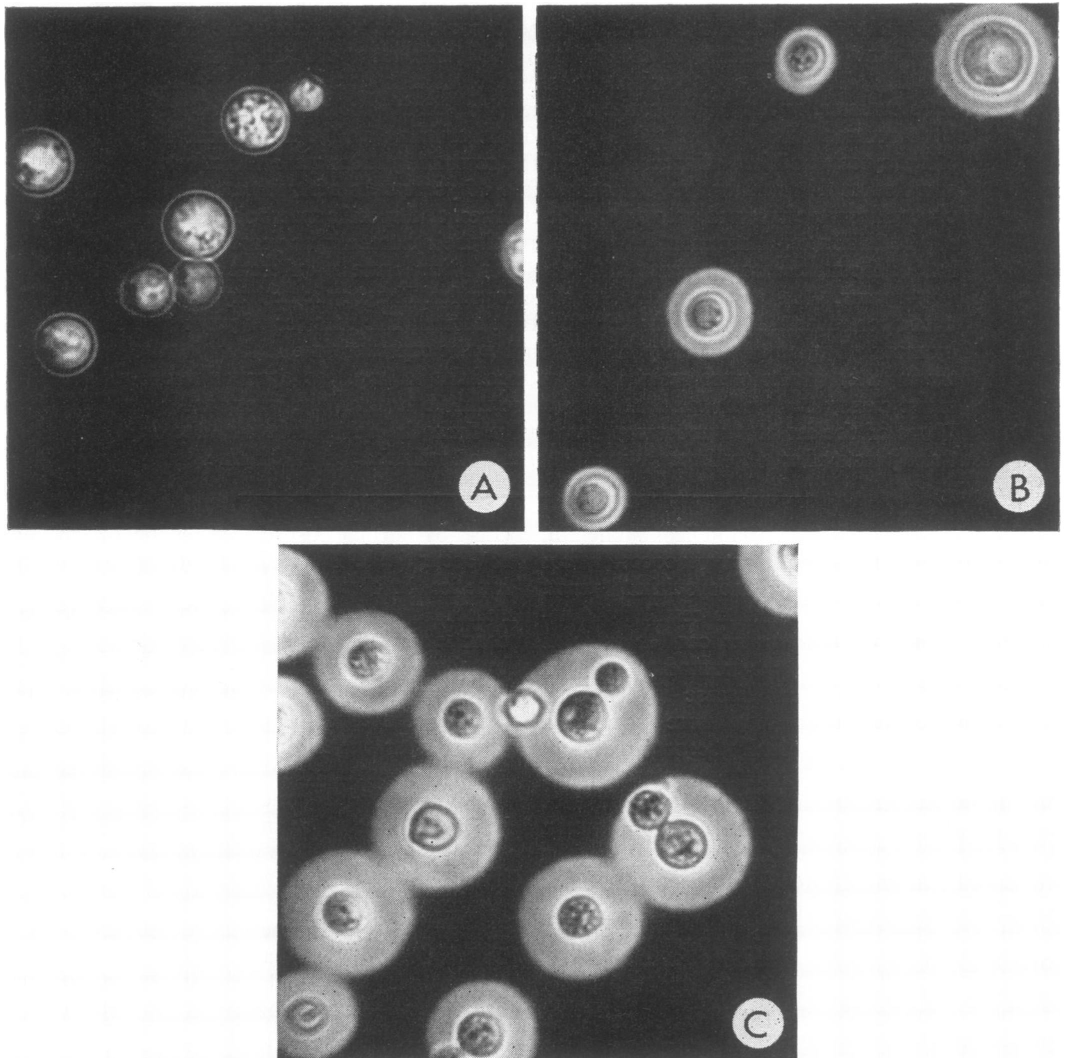

FIG. 1. *India ink negative staining of Cryptococcus neoformans (A) strain 15S, (B) strain 98M, and (C) strain 6L, illustrating capsules of small, medium, and large size, respectively.*

ml and 100 μg of streptomycin per ml, Grand Island Biological Co.). The cells were pelleted by centrifugation for 10 min at 220 × g, washed twice in HBSS, counted in a hemacytometer, and suspended at 5 × 10^6/ml in medium 199 (Microbiological Associates, Inc.) with sodium bicarbonate. Average yield was 15 million cells per rat, with greater than 80% viability, as determined by trypan blue dye exclusion. The cells were distributed to acid-washed, depyrogenated Leighton tubes with cover slips at a concentration of 10^6 cells per tube in medium 199 with 20% fresh isologous rat serum and incubated at 37 C. After 3 hr the macrophages had attached to glass; medium was replaced and cultures were reincubated for 24 to 36 hr.

Cryptococci were washed with medium 199 from the surface of slant cultures in the log phase, counted in a hemacytometer chamber, and, after aspiration of the old medium, added to macrophage cultures in 1 ml of medium 199 with 20% rat serum at a concentration of 5 to 10 cryptococci per macrophage. After 2 hr at 37 C, extracellular cryptococci were eliminated from all of the tubes by three washes with warm medium 199 without rat serum. At this time, the cover slip was removed from one or two randomly selected tubes, fixed in absolute methanol, and stained by the Wright procedure. The remaining tubes were replenished with warm medium 199 containing rat serum and reincubated for 24 or 48 hr, at which time one or two cover slips were removed for Wright staining. Three hundred macrophages were examined per slip; the per cent with ingested or cell-associated yeasts, the total number of intracellular or cell-associated yeasts, and the mean number of macrophages per field (as a reflection of cell density) were determined. To assay the survival of macrophages with time, the per cent change of macrophages per field at 24 and 48 hr was calculated by dividing the values for those periods by the number at 2 hr and multiplying by 100. Control tubes contained macrophages but no yeasts. In some experiments the yeast suspension was heat-killed at 55 C for 90 min before being added to the macrophages.

Every time a cover slip was sampled for staining, the slip from a companion tube was removed, inverted onto a slice of warm glucose-yeast extract-agar on a sterile microscope slide, and maintained in a humidified petri dish at 37 C. The "slide culture" was immediately examined by phase-contrast microscopy (Leitz Labolux with 40/0.65 objective and 12 × Periplan oculars) which enabled clear observation of macrophages and their ingested yeasts. The location of numerous fields on each culture was recorded by the use of a stage micrometer. A Polaroid camera attachment permitted the rapid production of an inexpensive print which assisted in subsequent location of specific fields. Forty to 200 macrophages per slip were recorded for number and location of specific, individual macrophages with ingested cryptococci; only phagocytes with a few intracellular yeasts were monitored to minimize any confusion about the viability of proximate cryptococci. The slide cultures were then incubated and reexamined intermittently for 6 to 48 hr. The macrophages retained enough integrity under these conditions to locate previously marked fields without difficulty. Viability of yeast cells was defined as ability to bud and multiply during the period of observation; there was no morphological distinction between live and dead yeast cells. Data were recorded as per cent phagocytes able to inactivate their ingested yeasts. Control tubes contained yeasts suspended in tissue culture medium with rat serum but lacking macrophages.

RESULTS

Phagocytosis. The morphology of the peritoneal exudate cells in tissue culture was typical (2); however, an incubation period of 24 to 36 hr was necessary before the entire population became pseudopodal. The spreading process developed more slowly than reportedly occurs with peritoneal exudate cells of other species (2). It was presumed that glass-adherent cells were viable and that rounding, loss of adhesiveness, and release of macrophages into the fluid medium were indications of cell destruction. When the washed cover slips were removed from Leighton tube cultures without yeasts, Wright-stained, respective fields counted, and the cell density estimated, there was consistently a decrease with time in the number of attached cells, viz. 95 and 80% survival at 24 and 48 hr, respectively. The per cent survival of macrophages in cultures exposed to cryptococci ranged from 70 to 90% and 40 to 70%, respectively, after 24 and 48 hr of exposure. No measurable differences in loss of macrophages were detected after exposure to yeasts with capsules of small, medium, or large size. When macrophages were exposed to heat-killed yeasts having capsules of medium size (98M and 110M), the loss of macrophages from cover slips was comparable to that observed with viable yeasts.

Table 1 shows the per cent of macrophages phagocytizing the various strains of *C. neoformans* and the numbers of ingested yeasts at various time intervals. There was an obvious inverse relationship between capsular size and extent of phagocytosis. After the initial 2 hr, the average number of cryptococci taken up per phagocytizing macrophage was 1.25 ± 0.07, 2.93 ± 1.62, and 5.70 ± 1.84, for the yeasts with large, medium, and small capsules, respectively. At this time, only 10 to 30 highly encapsulated yeasts, 80 to 375 moderately encapsulated yeasts, and 270 to 560 of the minimally encapsulated yeasts were engulfed per 100 macrophages. By then a substantial proportion (45 to 95%) of the macrophages had phagocytized the cryptococci with small or medium capsules, but considerably fewer (approximately 10 to 20%) ingested the yeasts with large capsules. In experiments with

TABLE 1. *Phagocytosis of variously encapsulated strains of Cryptococcus neoformans by Lewis rat macrophages*

Strain designation	Capsule size	Expt no.	Time after removal of extracellular yeasts (hr)	Per cent macrophages with ingested yeast[a]			No. of ingested yeasts per 100 macrophages[a]		
				Tube 1	Tube 2	Mean ±SE[b]	Tube 1	Tube 2	Mean ±SE[b]
110	Medium	18[c]	0	91	89	63.3 ± 16.8	203	223	172 ± 82
		19		54	66		139	183	
		20		52	52		83	94	
		21		45	47		98	102	
		26		72	66		309	281	
		18	24	93	93	78.0 ± 9.6	168	178	219 ± 115
		19		80	ND[d]		175	ND	
		20		65	71		156	173	
		21		69	ND		148	ND	
		26		82	78		450	423	
		18	48	97	94	84.5	329	320	302
		21		74	ND		279	ND	
145	Medium	11	0	81	95	78.3 ± 16.5	217	296	245 ± 85
		15		92	98		284	343	
		17		60	54		91	152	
		26		79	67		301	274	
		15	24	79	92	74.5 ± 9.9	288	393	246 ± 90
		17		67	69		158	152	
		26		74	66		250	235	
98	Medium	14	0	80	ND	68.2 ± 12.8	309	ND	249 ± 102
		22		64	50		148	157	
		24		80	ND		373	ND	
		26		59	55		169	153	
		14	24	63	ND	70.4 ± 8.9	210	ND	254 ± 99
		22		75	58		141	134	
		24		69	ND		382	ND	
		26		85	80		322	266	
		14	48	63	ND		184	ND	
		24		68	ND		523	ND	
15	Small	13	0	79	ND	77.2 ± 6.2	407	ND	410 ± 123
		23		73	74		278	270	
		25		71	88		553	543	
		23	24	68	50	74.3 ± 20.0	122	142	440 ± 376
		25		84	96		610	886	
		25	48	88	ND		587	ND	
6	Large	13	0	15	ND	15.9 ± 3.7	18	ND	20 ± 5
		23		18	10		21	13	
		25		21	16q		27	22	
		23	24	8	6	21.8 ± 18.6	16	8	33 ± 29
		25		45	37		73	37	
		25	48	50	ND		108	ND	

[a] Calculated from examination of 300 macrophages per Wright-stained cover slip per tube. The average number of phagocytized yeasts and the per cent of phagocytic cells in the tally of each 100 macrophages (counted in the same three representative sections of each cover slip), comprising the total of 300, did not deviate more than 20 and 10%, respectively.

[b] This is the mean of the one or two (averaged) slips assayed from each experiment within the time period.

[c] Identically numbered experiments indicate same lots of macrophages used.

[d] ND, the cover slip from only one tube was removed at that time period.

the yeasts with small and medium capsules, maximal levels of ingestion (70 to 95% of the macrophages) were usually reached within 2 hr, and little change occurred at 24 and 48 hr; but in cases where the initial percentage was lower (45 to 65%), increases were later observed (see experiments 17, 20–22, and 26 with 98M). Since the technique showed good reproducibility of data between cover slips from duplicate tubes, the observed variation among replicate experiments probably reflected an actual variability in the phagocytic capacity of different macrophage populations.

In some of the experiments, there was an increase in the per cent of macrophages with ingested yeasts and the number of intracellular yeasts at 24 and 48 hr. In others, there was a decrease. Both the absolute and per cent increase varied, however, from one experiment to another. An increase with time in the number of phagocytized cryptococci might be interpreted as evidence of intracellular growth of these yeasts, as opposed to multiplication of residual extracellular yeasts resulting in additional phagocytosis (assuredly, all of these were removed at the 2-hr washing). However, some of the increased count was undoubtedly due to loss of macrophages from the glass, release of their intracellular yeasts, and subsequent rephagocytosis. A decrease of intracellular census (see experiments 14, 18, 22, 23 and 26 with 145M) can also be attributed to the loss of phagocytes from cover slips. An analysis of the decrease in cell density in each experiment showed that the loss of macrophages from glass provided a plausible explanation for the corresponding change in the per cent of macrophages with ingested yeasts. Despite the difficulty of the variables inherent in this system, the mean values reflected no change, suggesting a lack of any significant degree of intracellular multiplication.

If the rat serum in the tissue culture medium were previously heated at 56 C for 30 min, there was a complete abrogation of phagocytosis. Table 2 exemplifies this effect with data for a strain with capsules of medium size. In these experiments the cryptococci were heat-killed and the extracellular yeasts were not removed after 2 hr. Even with continued exposure to yeasts for 48 hr or longer, phagocytosis was inhibited in the tubes with heated serum. Both viable and heat-killed cryptococci were equally phagocytizable with unheated serum, but neither was taken up in the presence of inactivated serum. This heat-labile opsonin was obligatory for phagocytosis of all the strains tested, it was preserved by storage at −70 C for up to 6 months, and it was not required during the preincubation of macrophages without yeast.

TABLE 2. *Phagocytosis of heat-killed Cryptococcus neoformans strain 145M by normal rat macrophages in the presence of 20% fresh, isologous serum, unheated or heated 56 C for 30 min*

Expt no.	Normal rat serum	Time of exposure of macrophages to yeast[a] (hr)	Per cent macrophages with ingested yeast[b]		No. of ingested yeasts/100 macrophages[b]	
			Tube 1	Tube 2	Tube 1	Tube 2
5[c]	Unheated	1	44	40	54	43
16		1	57	ND[d]	85	ND
5		24	42	47	78	70
12		24	65	57	131	91
16		24	59	ND	106	ND
5	Heated	1	0	1	0	1
16		1	1	ND	1.3	ND
5		24	0	1	0	1
12		24	1	0	1	0
16		24	1	ND	0.3	ND
5		48	0	3	0	4

[a] Extracellular yeasts were not rinsed out of Leighton tubes in these experiments.

[b] Calculated from examination of 300 macrophages per Wright-stained cover slip per tube.

[c] Identically numbered experiments indicate same lots of macrophages used.

[d] ND, cover slip from only one tube was removed at that time period.

Fate of ingested cryptococci. The agar slide culture technique unequivocally, although semiquantitatively, demonstrated the fate of engulfed yeast cells in situ. Whereas most of the macrophages (60% or greater) were unable to kill their phagocytized yeasts, definite proportions of the macrophage population were capable of inactivating ingested yeasts of certain strains, viz. 98M, 6L and 15S (Table 3). With strains 110M and 145M, however, there was only a negligible loss of ability to multiply. There were other definite and characteristic strain differences. At 0 hr, only 6% of the macrophages, at the most, contained nonviable cells of strain 110M or 145M, whereas up to 22% (average 9%) of the phagocytes killed intracellular cells of strain 98M; 4 to 31% (average 12%) killed strain 15S; and 7 to 39% (average 21%) killed strain 6L. With the exception of the experiments with the slightly and the highly encapsulated strains, and a single experiment with strain 98M, the extent of cryptococcidal phagocytes remained relatively unchanged at 0 and 24 hr, so apparently

TABLE 3. *Capacity of normal Lewis rat macrophages to inhibit the growth of various encapsulated strains of Cryptococcus neoformans after phagocytosis, as determined by slide culture on agar*

Strain	Capsule size	Expt no.	Per cent of phagocytes containing nonmultiplying yeast at hr after removal of extracellular yeasts[a]			
			0 Hr		24 Hr[b]	
			Tube A	Tube B	Tube C	Tube D
110	Medium	18[c]	4	ND[d]	11	6
		19	4	ND	6	ND
		21	3	ND	4	ND
		26	4	3	6	4
145	Medium	15	1	0	0	1
		17	1	0	2	2
		26	6	4	6	14
98	Medium	14	ND	ND	22	ND
		22	22	ND	20	19
		24	8	9	10	ND
		26	4	2	13	25
15	Small	13	5	31	27	56
		23	15	ND	58	ND
		25	16	5	35	25
		28	4	9	29	37
		29	6	17	17	44
6	Large	13	39	7	43	15
		23	ND	ND	33	ND
		25	31	23	26	32
		28	22	16	28	36
		29	17	12	35	40

[a] Forty to 200 phagocytes, most of which contained only one ingested yeast, were selected for observation per cover slip.

[b] In experiments where tubes were sampled at 48 hr, the per cent of phagocytes with nonviable intracellular yeast was 15 for strain 110M (experiment 18); 38, 31, and 44, strain 98M (experiments 14, 22, and 24, respectively); 23, 59, and 27, strain 15S (experiments 25, 28, and 29); and 34, 54, and 62, strain 6L (experiments 25, 28, and 29).

[c] Identically numbered experiments indicate the same lots of macrophages used.

[d] ND, a macrophage culture tube was not sampled at the time.

whatever macrophages are equipped to inactivate a given strain becomes evident very soon after the organisms are phagocytized. The yeasts with small capsules differed in that there was about a twofold increase in per cent of phagocytes showing the killing effect at 24 hr. With the highly encapsulated strain 6L, the magnitude of the increase at 24 hr was not consistent. In most cases, as already mentioned, the macrophages selected for observation contained only one intracellular yeast, which was necessary to eliminate any confusion about the viability of contiguous yeast cells and to permit a more equitable comparison between strains. Nevertheless, it appeared from observations of macrophages multiply infected that the number of intracellular yeasts did not seem to influence the fate of any one of them. Controls for each experiment showed no loss in viability of any cryptococci in the presence of tissue culture medium alone.

DISCUSSION

The results have shown that in vitro phagocytosis of different cryptococcal strains by rat peritoneal exudate cells occurs promptly. The number of yeasts ingested after exposure for 2 hr (Table 1, 0 time) confirms the observations of others (4, 5, 7) that phagocytosis and capsular size are inversely related. We have noted, however, in as yet unconfirmed experiments, that, when the exposure time was extended to 6 hr, phagocytosis of the strain characterized by large capsules increased to 71%, a level comparable to that attained in 2 hr with yeasts having small or medium capsules (Table 1). The slower rate of ingestion of the cells with large capsules, which possess greater surface area, suggests that particle size may be a factor in limiting phagocytosis. Another possibility is that the greatly encapsulated yeasts, possessing greater size but less density, settle through the 2- to 3-mm depth of medium onto the macrophage monolayer at a slower rate than yeasts with smaller capsules. This difference in settling rate may account, to some extent, for the differences in uptake of the two yeast types. Bulmer and Sans (5) were able to show with their phagocytic system that the addition of extracted and purified capsular polysaccharide specifically inhibited the normally high phagocytosis of a mutant having small capsules (5); however, inhibition under such circumstances could be explained on the basis of opsonin depletion, viscosity, ionic effects, etc. In fact, the macrophages might be exposed to greater amounts of polysaccharide after engulfment of several cells with small or medium sized capsules than after ingestion of only a few cells with large capsules.

With a slide culture technique, it was possible to observe visually the fate of engulfed cryptococci. Susceptibility to intracellular killing seemed to be a strain characteristic. Although the magnitude of difference between killing of different strains (Table 3) was not large, the reproduction, with few exceptions, of comparable values for

replicative experiments enforces the interpretation that strains of *C. neoformans* differ in their resistance to intracellular killing. It may be concluded that strains 110 and 145, both with medium sized capsules, were more refractory to killing by macrophages than strains 98M, 15S, and 6L. The fate of these yeasts after phagocytosis could not be correlated with the size of their capsules.

The data obtained by direct visualization (Table 3) suggest that most of the yeast cells, regardless of strain or capsular size, remain viable after ingestion. Yet the data of Table 1, derived from Wright-stained cover slips, imply a lack of substantial, intracellular multiplication. It is therefore probable that the macrophages exert a fungistatic effect on all the phagocytized cryptococci, with a distinct but limited ability to kill cells of certain strains of *C. neoformans* more than others. However, both techniques require some evaluation. For example, the total number of phagocytized yeasts seen in Wright stains proved to be a more sensitive index of phagocytic activity than the per cent of macrophages, in the same kind of preparation, having ingested yeasts because the former measurement evinced a difference among the three capsular sizes. The per cent of macrophages with ingested yeasts after exposure for 2 hr (Table 1, 0 time) was less with the strain having the largest capsule than with the other strains, but no distinction could be made between the yeasts with medium and small capsules. However, neither the per cent of phagocytizing macrophages nor the total number of phagocytized yeasts in the Wright-stained preparations (Table 1) could be correlated with the fate of a strain after phagocytosis as seen in situ (Table 3). As stated above, the overall inhibition of intracellular growth probably explains this lack of correlation adequately. Neither technique was able to distinguish the effects of any of several variables, such as loss of macrophages with and without ingested yeasts from glass and the extent of multiplication and rephagocytosis of the extracellular cryptococci liberated by destruction of these macrophages.

The method of measuring survival of macrophages was a relatively insensitive procedure which involved estimation of cell density from representative counts on the Wright-stained slips. A decrease in cell density was interpreted to indicate a loss of macrophages. By this technique, the loss of macrophages was similar regardless of capsular size of the yeasts to which the macrophages were exposed. That is, the loss of macrophages after 24 hr of exposure to cryptococci varied from 10 to 30% (which was distinctly greater than the 5% loss of macrophages without yeasts), but the variation was not correlated with strain differences. With a more precise method of quantitating macrophage census, perhaps differences in the effect of variously encapsulated yeasts upon the macrophages may have been observed. Similarly, the heat-killed yeasts also evidenced toxicity within this range, which would suggest that the macrophages were able to inhibit the multiplication of viable cryptococci, assuming that cytotoxocity is proportional to concentration of yeasts. Our observation that cryptococcal cells did not have a grossly deleterious effect on the survival of cultured macrophages seems to conflict with the findings of Gentry and Remington (8) that murine macrophages, cultured in monolayers and infected with cryptococci similarly to the method described herein, were destroyed within 48 hr, or sooner, if they are not "activated" by prior infection with *Listeria* and *Besnoitia*. Their phagocytic model differed, however, from ours in source of macrophages, culture medium, strain of *C. neoformans*, and the use of noninduced macrophages. Furthermore, Gentry and Remington failed to mention survival of uninfected control cells or to present quantitative data, so precise comparison of results is difficult. It is plausible that these technical variations could accoount for disparate results [e.g., Ralston and Elberg (16)]. Nevertheless, the rounding and gradual loss of macrophages that we observed in cryptococcal-infected cultures might have been indicative of the advent of an eventual cytotoxocity which would manifest itself more fully at a later time or under somewhat different conditions.

Our observation of a heat-labile serum factor essential for phagocytosis seems similar to the heat-labile serum opsonin indispensable for cryptococcal "ring formation" by rabbit peritoneal neutrophils and monocytes, as reported by Shahar et al. (18). This effect of the serum could be nonspecific, analogous to the phagocytosis-promoting factors described for certain bacterial systems which may include components of complement. Another possibility is the presence of a natural antibody which may be normally cytophilic. Cline and Lehrer (6) found that peripheral human monocytes required serum opsonins for phagocytosis of either *Candida albicans* or *C. neoformans*. Testing further with *C. albicans* alone, they found that purified immunoglobulin G promoted phagocytosis and concluded that complement was probably not involved. With regard to the possibility of a heat-labile natural antibody, Weir and Elson (19) have shown that the Fc portion of

rat immunoglobulin M agglutinins to sheep red cells was destroyed by treatment at 56 C for 30 min; the heated antiserum lost the ability to bind complement (hemolysis) but retained specific antigen-combining activity (hemagglutination). It is not, therefore, unreasonable to speculate that a natural, cytophilic antibody, undetectable by conventional serological tests, might have been present in the normal serum and have lost its ability to bind to macrophages after heating; it must be assumed that the cultured macrophages had lost any previously attached cytophilic antibody, by dilution or elution, during harvest at 0 C or preincubation at 37 C. At any rate, in an effort to further delineate the specificity and chemical nature of this factor, controlled tests for phagocytic activity with absorbed serum, diluted serum, serum fractions, opsonized cryptococci, and exogenously supplied complement should be conducted.

The technique described herein for measurement of phagocytosis and killing of cryptococci may be applied to the investigation of additional areas, such as a comparison of strain pathogenicity in vivo with susceptibility to macrophages in vitro, the effect of cryptococcal agglutinins, or the activity of macrophages from immunized or infected animals. Admittedly, however, the in vitro system would seem a more natural one if alveolar instead of peritoneal macrophages were used, since the two are known to differ in morphology and metabolism and the former possess higher levels of degradative enzymes (15). Peritoneal macrophages, however, are more often studied, are simpler to harvest, and may provide a more versatile system in that their inhibition of migration is a probable correlate of delayed-type hypersensitivity (3). Furthermore, Gadebusch and Johnson (7) demonstrated identical levels of cryptococcal phagocytosis by both rabbit alveolar macrophages and peritoneal exudate cells.

ACKNOWLEDGMENTS

The senior author was a trainee in medical mycology. This investigation was supported by Public Health Service training grant 5-TO1-AI00003 from the National Institute of Allergy and Infectious Diseases.

We are indebted to Agatha Sims for technical assistance.

LITERATURE CITED

1. Baker, R. D., and R. K. Haugen. 1955. Tissue changes and tissue diagnosis in cryptococcosis. Amer. J. Clin. Pathol. 25:14–24.
2. Bennett, B. 1967. Comparative morphology of macrophages in tissue culture, p. 74–84. *In* DiLuzio-Paoletti (ed.), The reticuloendothelial system and atherosclerosis. The Plenum Press, New York.
3. Bloom, B. R., and B. Bennett. 1968. Migration inhibitory factor associated with delayed-type hypersensitivity. Fed. Proc. 27:13–15.
4. Bulmer, G. S., and M. D. Sans. 1967. *Cryptococcus neoformans*. II. Phagocytosis by human leukocytes. J. Bacteriol. 94:1480–1483.
5. Bulmer, G. S., and M. D. Sans. 1968. *Cryptococcus neoformans*. III. Inhibition of phagocytosis. J. Bacteriol. 95:5–8.
6. Cline, M. J., and R. J. Lehrer. 1968. Phagocytosis by human monocytes. Blood 32:423–435.
7. Gadebusch, H. H., and A. G. Johnson. 1966. Natural host resistance to infection with *Cryptococcus neoformans*. V. The influence of cationic tissue proteins upon phagocytosis and on circulating antibody synthesis. J. Infect. Dis. 116:566–572.
8. Gentry, L. O., and J. S. Remington. 1971. Resistance against *Cryptococcus* conferred by intracellular bacteria and protozoa. J. Infect. Dis. 123:22–31.
9. Gordon, M. A., and D. K. Vedder. 1966. Serologic tests in diagnosis and prognosis of cryptococcosis. J. Amer. Med. Ass. 197:961–967.
10. Goren, M. B. 1967. Experimental murine cryptococcosis: effect of hyperimmunization to capsular polysaccharide. J. Immunol. 98:914–922.
11. Littman, M. L., and J. E. Walter. 1968. Cryptococcosis: current status. Amer. J. Med. 45:922–932.
12. Louria, D. B., and T. Kaminski. 1965. Passively-acquired immunity in experimental cryptococcosis. Sabouraudia 4:80–84.
13. Louria, D. B., T. Kaminski, and G. Finkel. 1963. Further studies on immunity in experimental cryptococcosis. J. Exp. Med. 177:509–520.
14. Miller, D. G. 1965. Hodgkin's disease, lymphosarcoma, and chronic lymphocytic leukemia, p. 382. *In* M. Samter and H. L. Alexander (ed.), Immunological diseases. Little, Brown & Co., Boston.
15. Pearsoll, N. N., and R. S. Weiser. 1970. The macrophage. Lea and Febiger, Philadelphia.
16. Ralston, D. J., and S. S. Elberg. 1971. Medium-dependent activity of immune serum on *Brucella*-infected macrophages. Infect. Immunity 3:361–362.
17. Schneerson-Porat, S., A. Shahar, and M. Aronson. 1965. Formation of histiocyte rings in response to *Cryptococcus neoformans* infection. J. Reticuloendothel. Soc. 2:249.
18. Shahar, A., Y. Kletter, and M. Aronson. 1969. Granuloma formation in cryptococcosis. Israel J. Med. Sci. 5:1164–1172.
19. Weir, D. M., and C. J. Elson. 1968. Heat labile antibody in rat serum. Clin. Exp. Immunol. 3:725–731.
20. Zimmerman, L. E., and H. Rappaport. 1954. Occurrence of cryptococcosis in patients with malignant disease of the reticulo-endothelial system. Amer. J. Clin. Pathol. 24:1050–1072.