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Phagocytosis of *Cryptococcus neoformans* by Alveolar Macrophages

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Guinea pig pulmonary macrophages phagocytized but did not kill nonencapsulated cells of *Cryptococcus neoformans*. The phagocytic process was inhibited by cryptococcal capsular polysaccharide. Pulmonary macrophages, activated by preinjecting heat-killed bacteria into intact animals, did not kill the engulfed yeast cells. Labeled cells of *C. neoformans* were neither killed nor cleared from guinea pig lungs 6 h postexposure. The results of our experiments indicate that during the first few hours after the lung is exposed to the infectious particle of *C. neoformans* the pulmonary macrophage does not function primarily to kill engulfed yeast cells. We believe that a rapid yet transient acute inflammatory response probably plays a major role in this process during the first few hours after *C. neoformans* enters the lung.

Earlier studies have shown that the capsule of the yeast *Cryptococcus neoformans* is antiphagocytic (6). We have proposed that this polysaccharide be classified as one of the few described virulence factors in fungi pathogenic to humans (4). However, other evidence (9) strongly suggests that the infectious particle for naturally occurring cryptococcosis is not an encapsulated organism, but a nonencapsulated yeast less than 5 μm in diameter. When this particle enters the lung, suitable conditions are probably present for the production of antiphagocytic capsular material within a few hours (9). Before any definitive conclusions can be drawn about the entire pathogenesis of cryptococcosis, however, phagocytosis experiments must be carried out in which alveolar macrophages rather than polymorphonuclear leukocytes (PMN) are used, as alveolar macrophages would be expected to be the first line of defense encountered by the entering cryptococci.

We report here on phagocytosis experiments with alveolar macrophages. Our studies were conducted to determine whether these cells phagocytize *C. neoformans*, what constitutes optimal conditions for phagocytosis, and whether the phagocytized yeast cell is killed.

MATERIALS AND METHODS

Information has been published on the following procedures: source and maintenance of cultures (4); preparation of low-pH and high-pH media for culturing *C. neoformans* (strain CIA) in a nonencapsulated and normally encapsulated state, respectively (9); phagocytosis protocol (5); isolation of cryptococcal capsular material (ACIA; 9); and determination of the intracellular fate of phagocytized yeast cells (15).

Animals. Adult male and female Hartley strain guinea pigs were used throughout the study as the source of alveolar macrophages for in vivo isotope experiments and production of activated macrophages.

Organisms. Nonencapsulated and encapsulated phases of *C. neoformans* (strain CIA) were used in these studies. The bacteria *Escherichia coli* (ATCC 11775), *Salmonella typhosa*, and *Serratia marcescens* (stock collection, University of Oklahoma Health Sciences Center, Department of Microbiology and Immunology) were also used.

Preparation of radioactively labeled organisms. A special medium was devised for growing small nonencapsulated *C. neoformans* cells for radioactive labeling and transtracheal instillation experiments. The medium was a modification of Czapek-Dox broth (Difco), hereafter referred to as Mc broth, containing the following components: $MgSO_4$, 0.5 g; KCl, 0.5 g; $FeSO_4$, 0.01 g; K_2HPO_4, 0.5 g; yeast extract, 1.0 g; peptone (Difco), 5.0 g; and distilled water, 1 liter (pH 5.5; adjusted with 1.0 N HCl).

Depending on the particular experiment, two 250-ml flasks (one for back-up) containing 50 ml of Mc broth were inoculated with 2.0×10^6 cells of nonencapsulated *C. neoformans* that had been starved for 48 h in a pH 7.0 tris(hydroxymethyl)-aminomethane buffer. One or two μCi of $^{32}H_3PO_4$ in 0.02 N HCl was added to each flask. (Mc broth promotes growth of yeast cells with no capsules and a mean cell diameter of 4.0 μm [range, 3.6 to 4.7 μm].) The cultures were placed on a mechanical shaker and incubated at room temperature. After 5 days, the organisms were harvested by centrifugation and washed four times in a pH 5.0 acetate buffer. The

washed, radioactive organisms were resuspended in Mc broth and counted microscopically with a hemocytometer. Samples of the organisms were dilution-plated to determine the number of viable yeast cells per milliliter.

Alveolar macrophages. Under aseptic conditions, each guinea pig, anesthetized with Nembutal, was incised from the cephalic part of the trachea to the xiphoid process. The trachea was dissected out and clamped shut. This killed the animal and prevented backwash of normal microbial flora from the posterior pharynx. The intact lungs and trachea were quickly removed from the thoracic cavity and washed vigorously with sterile 0.85% NaCl. Then the clamp on the trachea was removed and the lungs were filled, via the trachea, with cold (4 C), sterile Eagle balanced salt solution (BSS). The trachea was again clamped shut, and the fluid-filled lungs were gently massaged for about 2 min. After the clamp was again removed, Eagle solution containing the alveolar macrophages was allowed to flow into 15-ml sterile, siliconized centrifuge tubes. The first washing from the lungs was discarded because it contained predominantly so-called "dust cells" (alveolar macrophages metabolically in death phase and containing dust particles) and other cellular debris. Cells from subsequent washings were washed three times in Eagle BSS at a centrifuge speed not exceeding $400 \times g$. After the final centrifugation, the cells were resuspended in cold Eagle BSS, counted, and stained with 1% trypan blue to determine viability.

Electron microscopy. In an attempt to demonstrate yeast cells phagocytized by alveolar macrophages, samples from in vitro fate experiments were centrifuged at $120 \times g$ to form a pellet. The supernatant fluid was decanted and the pellet was suspended in 0.85% sterile saline.

The alveolar macrophages were recentrifuged and resuspended in physiological sucrose buffer for 20 min. After recentrifugation for pellet formation, each sample was fixed in osmium for 20 min and then was washed and resuspended in Veronal buffer. Next, each sample was dehydrated in graded ethyl alcohols: two changes of 70% for 10 min, two changes of 95% for 15 min, and two changes of 100% for 20 min. Each sample was then resuspended in propylene oxide for two changes of 10 min each before being placed in a 50:50 mixture of propylene oxide and Epon 812 for 1 h. After the samples were removed from the 50:50 mixture and allowed to remain in pure Epon 812 for 1 h, they were divided into two parts and put into gelatin capsules. One part was embedded in Epon 812 and the other part (back-up) was embedded in Araldite. The embedded samples were allowed to polymerize at 60 C overnight. Sectioning was performed on a Sorvall Porter-Blum ultramicrotome, model MT-1, with glass knives broken on an LKB knife breaker. Thin sections were placed on #300 mesh copper grids and stained with saturated uranyl acetate and lead citrate. Sections were examined and photographed on an RCA EMU-3F electron microscope, employing a voltage of 100,000 kV.

In vivo fate of ^{32}P-labeled organisms. Radioactive organisms grown in Mc broth were introduced aseptically into guinea pig lungs. The trachea was exposed surgically, and viable yeast cells were injected into the lungs via the trachea. Best results were achieved when the organisms were introduced in a concentrated form (1.5×10^7 to 1.7×10^7 cells) during inspiration.

Animals were sacrificed at various periods up to 6 h postinstillation. Lungs were removed by a technique similar to that for collecting alveolar macrophages. Each set of lungs was dissected free from the trachea and main-stem bronchi and was homogenized in 15 ml of cold Mc broth or cold Eagle BSS. Samples of the homogenate were dilution-plated into Sabouraud dextrose agar. Three days later, the plates were examined to determine the number of *C. neoformans* colonies. The remaining homogenate was counted on a liquid scintillation counter (Nuclear-Chicago Corp., Des Plaines, Ill.). Three guinea pigs were tested for each period of the transtracheal instillation experiments.

Activated alveolar macrophages. Activated macrophages apparently have a large complement of lysosomal enzymes and so may be able to kill ingested organisms with increased efficiency (2). Guinea pig alveolar macrophages were activated by treating animals as follows. Three guinea pigs were inoculated intravenously with 3.0×10^6 heat-killed *Salmonella typhosa* cells, and another three animals were inoculated with 3.0×10^6 heat-killed *Serratia marcescens* cells. Ten days later, the animals were sacrificed, and their lungs were excised to provide a source of activated macrophages. Previously described fate studies were made with these alveolar macrophages to determine their ability to kill *C. neoformans*. Acid phosphatase and β-glucuronidase levels were also determined and compared with those of normal alveolar macrophages. These enzymes are usually elevated in activated macrophages (2). Acid phosphatase and β-glucuronidase levels from alveolar macrophages were determined by the procedure in the Worthington Biochemical Manual (Worthington Biochemical Corp., Freehold, N.J.). Lysosomes, the source of the enzymes, were extracted from the alveolar macrophages by the method based on McRipley and Sbarra's procedure for PMN (12).

RESULTS

In vitro phagocytosis studies. Results of initial phagocytosis experiments indicated that guinea pig alveolar macrophages could phagocytize nonencapsulated cells of *C. neoformans* within 15 min. On the basis of this observation, a study was undertaken to discover the biological conditions necessary to achieve maximal phagocytosis in vitro. Since serum promotes engulfment in many in vitro and in vivo situations, it was decided to determine whether a particular concentration of serum provided maximal phagocytosis of *C. neoformans*. Control cells, without serum, had only 18% phagocytosis; the system containing 65% autologous serum had approximately 48% phagocytosis. The system containing 45% serum had 52%

phagocytosis. The ratio of cells to alveolar macrophages in these experiments was approximately 3:1. Although the experiments were allowed to proceed for 120 min, maximal phagocytosis was recorded after 60-min incubation.

Once the optimal serum concentration was determined, the ratio of yeast cells to alveolar macrophages was varied. All ratios except the 1:1 ratio yielded good percent phagocytosis (Fig. 1). However, it is evident from the phagocytic index (number of yeast cells per macrophage) that the 2:1 and 4:1 ratios provided the best conditions for phagocytosis.

Effect of capsule polysaccharide on phagocytosis. Because cryptococcal polysaccharide is known to inhibit phagocytosis by human PMN (6), an experiment was devised to determine whether it also inhibits phagocytosis by alveolar macrophages. The usual phagocytic assay was done, with the use of 30% autologous guinea pig serum, alveolar macrophages, and nonencapsulated cryptococcal cells. To this system, various amounts of partially purified cryptococcal capsular polysaccharide were added. After 60-min incubation, the percent phagocytosis was determined (Table 1). It required 100 μg of cryptococcal capsular material to reduce phagocytosis from 52 to 30%; the percent phagocytosis was reduced by half with the addition of 600 μg.

In vitro fate studies. An important objective of the work was to determine whether guinea pig alveolar macrophages could kill phagocytized cryptococcal cells. Figure 2 represents data from these experiments. The results indicate that 33.9% of the cryptococcal cells remained viable in the system with alveolar macrophages (AV/W). A similar percentage of yeast cells remained viable when macrophages were omitted from the system (AV/WO). As previously reported (15), decrease in yeast cell viability in such experiments can be attributed to the anticryptococcal serum factor. Trypan blue uptake by the guinea pig alveolar macrophages after 4-h incubation showed that 84% of these cells remained viable.

TABLE 1. *Inhibition of phagocytosis of guinea pig alveolar macrophages by C. neoformans capsular material*

Capsule material (μg/ml)	Percent phagocytosis
1,800	24
1,500	22
1,200	21
900	27
600	26
300	29
100	30
0	52

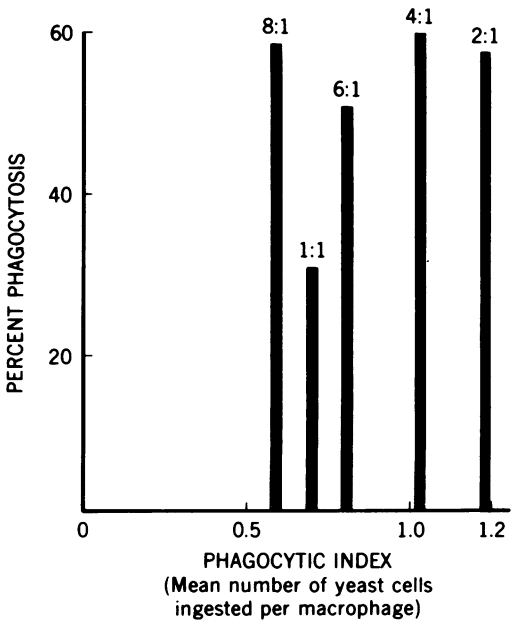

FIG. 1. *Effect of yeast cell and alveolar macrophage ratios on percent phagocytosis and phagocytic index.*

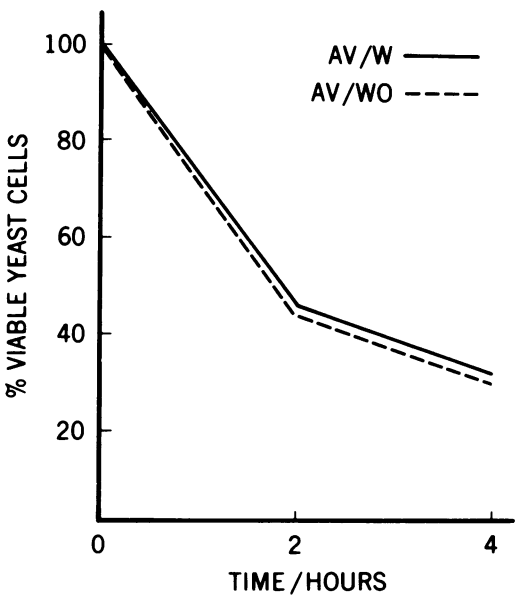

FIG. 2. *Guinea pig alveolar macrophage fate experiments: nonencapsulated C. neoformans incubated with (AV/W) or without (AV/WO) alveolar macrophages.*

To obviate the possibility that the alveolar macrophages were functionally inadequate, *E. coli* was tested in the phagocytic system. Only 4.6% of *E. coli* cells remained viable after 2-h incubation with the guinea pig alveolar macrophages.

Activated alveolar macrophages. It has been shown that macrophages can be activated by endotoxin and by gram-negative bacteria. Activated macrophages contain more lysosomal enzymes and, in certain instances, show increased bactericidal capacity. Table 2 shows the results of injecting heat-killed *S. typhosa* and *S. marcescens* into guinea pigs to activate their alveolar macrophages. Both organisms caused increases in enzyme activity per milligram of protein. In the animals injected with *S. typhosa*, acid phosphatase activity per milligram of protein increased from 96.0 to 128.7, and β-glucuronidase activity per milligram of protein increased from 27.3 to 33.6. Similar results followed the injection of *S. marcescens*. These activated alveolar macrophages were tested for their ability to kill nonencapsulated cryptococcal cells. Figure 3 shows the results of fate experiments with *S. marcescens*-activated macrophages. Note that after 4 h of incubation the presence or absence of activated alveolar macrophages made no observable difference in killing. Results were similar from macrophages activated with *S. typhosa*.

Electron microscopy showed no structural damage in cryptococcal cells phagocytized by guinea pig alveolar macrophages. A white ring, or halo, was observed frequently between cell walls and capsule material. According to Edwards et al. (8), this indicates that the cryptococcal cells were alive at the time of fixation.

In vivo fate studies. Since the in vitro fate studies demonstrated that guinea pig alveolar macrophages did not kill engulfed cryptococcal cells, experiments were devised to study in vivo fate. Radioactive cryptococcal cells were introduced into the lungs of live guinea pigs by transtracheal instillation. Approximately 1.5×10^7 to 1.7×10^7 viable cryptococcal cells and 2.0 cc of air were injected into the lower trachea of each animal. At selected times postinstillation, the animals were sacrificed. After lungs were removed, homogenized (as in previous experiments), and scored for radioactivity, the number of viable *C. neoformans* cells was determined. Table 3 shows that the average number of viable cryptococcal cells decreased from 1.51×10^7 to 1.24×10^7 per whole lung during the 360-min period of incubation. A reduction in viability of only 18% occurred after 6-h incubation, with a concomitant 14% decrease in ^{32}P counts. From these observations, it appeared that the cryptococcal cells were neither killed nor cleared from the lungs during this period.

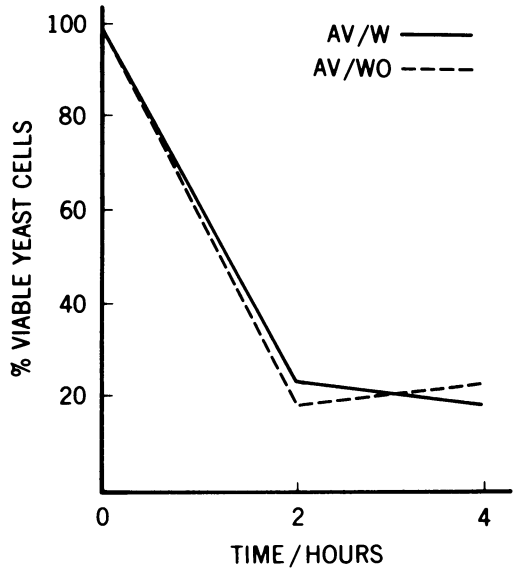

FIG. 3. *Activated macrophage fate experiment: nonencapsulated C. neoformans incubated with (AV/W) or without (AV/WO) guinea pig alveolar macrophages activated with heat-killed S. marcescens.*

TABLE 2. *Acid phosphatase and β-glucuronidase activity of normal and activated guinea pig alveolar macrophages*

Enzyme	Activity/mg of protein		
	Normal alveolar macrophages	*S. typhosa*-activated alveolar macrophages	*S. marcescens*-activated alveolar macrophages
Acid phosphatase	96.0	128.7	119.3
β-Glucuronidase	27.3	33.6	37.2

DISCUSSION

Findings in preceding studies led us to suggest that the infectious particle for cryptococcosis is probably a nonencapsulated yeast cell less than 5 μm in diameter (9). Since this organism resides in soils throughout the world, it would seem likely that it enters a potential host through trauma sites or puncture wounds. However, skin lesions are rarely seen in patients with cryptococcosis, and skin is not considered an important portal of entry in cryptococcosis. This might be accounted for in part by the potent anticryptococcal factor found in normal

TABLE 3. *Deposition and clearance of ^{32}P-labeled nonencapsulated C. neoformans from guinea pig lungs*

Time after instillation (min)	Animal no.	*C. neoformans*/ lung (cell count × 10^7)	^{32}P/lung (counts/min × 10^4)
0	1	1.64	1.43
0	2	1.43	1.35
0	3	1.51	1.39
	Avg	1.51	1.37
30	4	1.52	1.38
30	5	1.58	1.28
30	6	1.46	1.41
	Avg	1.52	1.36
90	7	1.53	1.30
90	8	1.47	1.30
90	9	1.49	1.24
	Avg	1.50	1.27
180	10	1.30	1.31
180	11	1.41	1.24
180	12	1.45	1.26
	Avg	1.38	1.27
360	14	1.31	1.23
360	15	1.21	1.16
	Avg	1.24	1.19

serum (3, 14) and by the fact that peripheral leukocytes readily phagocytize and kill nonencapsulated cells of *C. neoformans* (15). The effects of these factors are probably enhanced or supplemented by cationic proteins released from host cells. According to Gadebusch and Johnson (11), cationic proteins stimulate leukocyte emigration and adhesion and increase capillary permeability.

Because most authorities accept the lungs as the primary portal of entry for *C. neoformans*, we initially think in terms of an interaction between the alveolar macrophage and a potentially infectious particle. Such was our reasoning when we began this investigation.

Mitchell and Friedman (13) investigated phagocytosis of *C. neoformans* by rat peritoneal exudate cells and reported that most of the macrophages were unable to kill phagocytized yeast cells. Their findings indicated that phagocytosis was proportional to capsular thickness, but ability to kill was not influenced by capsule size; susceptibility to killing seemed to be largely strain-dependent. Diamond and Bennett (7) reported that macrophages cultured in vitro from human peripheral monocytes did not kill ingested cells of *C. neoformans*. They concluded that the monocyte-derived macrophage probably does not play a central role in human resistance to cryptococcosis. Gadebusch and Johnson (11) found that the percent phagocytosis of rabbit peritoneal exudate cells and of lung macrophages was similar (75%, 76%); they did not report on the fate of the phagocytized yeast particle.

We found that guinea pig alveolar macrophages phagocytize, in vitro, nonencapsulated cells of *C. neoformans*. This process was inhibited by microgram amounts of cryptococcal capsular material. Both in phagocytosis and in the inhibition of that process, guinea pig alveolar macrophages resemble peripheral human PMN; however, the similarity extends no further. The following differences have been observed between guinea pig lung macrophages and human PMN in the in vitro phagocytosis of cells of *C. neoformans*.

(i) Alveolar macrophages required serum for phagocytosis; PMN did not appear to (15).

(ii) The percent phagocytosis by lung macrophages was less than that recorded for PMN (5).

(iii) Lung macrophages did not kill ingested cells of *C. neoformans*, in sharp contrast to PMN. We were unable to demonstrate, with in vivo experiments, that the guinea pig lung was capable of either killing or clearing cells of *C. neoformans* up to 6 h postexposure. Attempts to demonstrate killing by activated macrophages (from animals previously injected with *Salmonella* or *Serratia* spp.) were negative. In one experiment (J. R. Tacker, Ph.D. thesis, Univ. of Oklahoma Health Sciences Center, Oklahoma City, 1974) with human alveolar macrophages, phagocytized cells of *C. neoformans* were not killed 1 h postingestion.

From the data reported here, we conclude that the pulmonary macrophage does not function by itself, if it functions at all, as a killing cell during the first few hours after the lung is exposed to the infectious particle of cryptococcosis. How then are these particles killed in the normal potential host? We presently know of several factors that could contribute to the processes of killing or elimination. Current investigations in this laboratory (M. Dublin, Ph.D. thesis, in preparation, Univ. of Oklahoma Health Sciences Center, Oklahoma City) demonstrate that normal human saliva contains a potent anticryptococcal system (probably myeloperoxidase). Certain groups of debilitated patients appear to have decreased amounts of this factor.

An important defense mechanism that is all too often overlooked is respiratory mucosa ciliary action. It seems probable that many potential infectious agents, including *C. neoformans*, are cleared by this mechanism before being deposited into the lung proper.

If an infectious particle of *C. neoformans* enters the lung milieu, how is it killed? Gadebusch and Johnson (11), in what must be one of the most neglected statements in cryptococcal literature, reported that the importance of inflammatory response in host resistance to cryptococcosis has been underrated. They observed a typical acute inflammatory response following peritoneal challenge with *C. neoformans*. Such a response would invoke a sequence of events similar to that occurring when *C. neoformans* enters the skin. This type of response has possibly been overlooked because it is not consistent with our concepts of the pathological responses in typical cases of cryptococcosis. But, just as there may be great differences in the meanings of "infection" and "disease," there also may be great difference between a host's responsiveness to initial contact with a potentially infectious particle and the host's response as seen in a well-developed disease state. In cryptococcosis it seems likely that the initial contact in the lung between the yeast cell and the normal host is one of acute inflammation. Since this inflammatory phase is brief and transient and precedes the infection phase, it would seldom be encountered. Perhaps, in the recent case reported by Farmer and Komorowski (10), this initial phase was prolonged and resulted in a disease state because of some unreported, underlying immunological abnormality. The patient in the study was infected with a capsule-deficient strain of *C. neoformans*. These workers reported that the capsule-deficient yeast isolated from the patient elicited an intense inflammatory response in mice. It was characterized by early suppuration and phagocytosis followed by marked histiocytic and fibroblastic reaction, limiting the infection. Little inflammatory response was noted when a normally encapsulated strain was used.

Earlier we reported that nonencapsulated infectious particles not killed within a few hours after entering the lung began to produce antiphagocytic capsular material (9). Demonstrating that capsular polysaccharide inhibits phagocytosis does not imply that encapsulated particles will not eventually (at a slower rate) be phagocytized. Mitchell and Friedman (13) indicated that encapsulated cells with prolonged incubation may reach the same percent phagocytosis as poorly encapsulated isolates. This means that, even if the initial lung defense mechanisms fail and the yeast begins to synthesize capsular material, it may still be phagocytized. If this process is accomplished solely by alveolar macrophages, it appears that the yeast cells will neither be killed nor cleared. A possible exception would be that in a highly susceptible host clearance could be one vehicle for dissemination. In the normal host the phagocytizing macrophage either must act in concert with other cells or factors to kill its inhabitants, or in some manner effect yeast cell stasis by compartmentalization (walling-off mechanisms). In this contained state yeast cells may die or may live for years at a reduced metabolic rate, in either normal forms or perhaps as protoplasts (1). If viability is retained, some future change may shift the host-parasite relation to the advantage of the yeast cells, and dissemination may follow. In such instances, clinical evidence of cryptococcosis may represent the conversion from a latent (concealed; not manifest) to a disease state. In our opinion, most cases of cryptococcosis diagnosed today may have followed this pattern.

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