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EVENTS RELATED TO THE PHAGOCYTOSIS OF *CRYPTOCOCCUS NEOFORMANS* BY ALVEOLAR MACROPHAGES

A DISSERTATION

SUBMITTED TO THE GRADUATE FACULTY

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EVENTS RELATED TO THE PHAGOCYTOSIS OF *CRYPTOCOCCUS NEOFORMANS* BY ALVEOLAR MACROPHAGES

APPROVED BY

DISSERTATION COMMITTEE

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EVENTS RELATED TO THE PHAGOCYTOSIS OF CRYPTOCOCCUS NEOFORMANS BY ALVEOLAR MACROPHAGES

CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

Cryptococcosis is a subacute or chronic human infection caused by the yeast Cryptococcus neoformans. The organism has a pronounced predilection for the central nervous system, although it may cause a subacute or chronic infection of the skin, lungs, or other parts of the body. A complete and accurate review of the literature concerning the history of cryptococcosis has been presented by Price (1971).

Several years ago Bulmer and co-workers started an investigation of the pathogenesis of cryptococcosis. These studies began with work on the properties of C. neoformans, which make it a pathogen and evolved into studies of host defense mechanisms against the invading organisms (Bulmer, Sans, and Gunn, 1967; Bulmer and Sans, 1967; Bulmer and Sans, 1968; Farhi, Bulmer, and Tacker, 1970; Tacker, Farhi, and Bulmer, 1972).

For several years, it has been assumed that the respiratory tract is the portal of entry in man for Cryptococcus neoformans. This assumption has been based, mainly, on reported cases of primary pulmonary cryptococcosis and on isolation of the organisms from soils and bird droppings from which it can be aerosolized (Powell, Dahl, Weeks, and Tosh, 1972; Smith, Ritter, Larsh, and Furcolow, 1964; Ritter and Larsh, 1963; Khan, Myers, and Koshy, 1959; Taylor, 1970; Emmons, 1955; Litman and Zimmerman, 1956; Kao and Schwarz, 1957). If one assumes these data to be valid, then it becomes of interest to learn more about the fate of organisms which lodge in the lungs. To do this, one must consider the elements of the lung and how the lung is able to clear itself of the foreign material.

Because pulmonary macrophages engulf inhaled dust particles and can often be identified in the sputum as carriers of inhaled, insoluble foreign materials, they have long been considered an integral component of the pulmonary clearance mechanism. Yet, recognition of the fact that pulmonary macrophages may be specific entities and may play a specific role in host defense mechanisms is relatively recent.

The importance of pulmonary macrophages as a lung clearance mechanism was reaffirmed by the publications of La Belle and Brieger (1959, 1960). They found that the amount of dust eliminated from the lungs during the early post-exposure period was proportional to the numbers of free

phagocytic cells present in a saline lavage of the lungs, and to the number of phagocytes visible in lung sections per unit area. The authors concluded that "transport of deposited particulates by phagocytes is the primary mechanism by which inhaled dust particles are eliminated from the lung."

Myrvik, Leake and Fariss (1961 b) were able to collect large numbers of rabbit alveolar mononuclear cells which resembled macrophages and which were capable of phagocytizing particulate material in vitro. Cell free extracts were prepared by alternate freezing and thawing or by sonic oscillation. The extracts contained large amounts of lysozyme (2,000 to 4,000 µg/ml of packed cells), which indicated that the cells might be different from other monocytes. The authors also suggested, based on their high state of purity (less than 1 per cent polymorphonuclear cells), that these cells were the "normal scavenger flora of the lower respiratory tract."

At about the same time that Myrvik, Leake and Fariss (1961 a) reported a metabolic difference between alveolar macrophages and other white blood cells, the excellent review by Karnovsky (1962) was published. Until that time, very little information was known concerning the metabolic basis of phagocytosis in mammalian phagocytic cells, other than in polymorphonuclear cells and in monocytes. Karnovsky (1962) also made a comparison using guinea pig polymorphonuclear

leucocytes and monocytes (obtained from the peritoneum), of metabolic activity during phagocytosis. In addition, information on the metabolic activity of lung macrophages was obtained. The metabolic results may be summarized as follows: Polymorphonuclear leucocytes, monocytes, and alveolar macrophages have a "resting" uptake of oxygen of approximately 2.5, 7.5, and 30 µl/hr/mg protein, respectively. Respiration of polymorphonuclear leucocytes, monocytes, and alveolar macrophages increases about 2.5 fold, 3.5 fold, and only 20 per cent respectively, during phagocytosis. Using glucose labeled in carbon-1 or carbon-6, the ratio (C1:C6) of counts appearing in expired CO_2 of polymorphonuclear leucocytes increased from a value of 8, at rest, to 22. With monocytes, the ratio at rest is rather high (approximately 20) and is unchanged by phagocytosis. The ratio for alveolar macrophages is also unchanged by phagocytosis, remaining about 8, with the appearance of label in the CO_2 from each type of labeled glucose: In other words phagocytosis caused an increase of less than 20 per cent which was proportional to aforementioned oxygen uptake. The influence of various metabolic inhibitors on the three types of phagocytes was of considerable interest. The principle observation was that phagocytosis in all of the cell types is inhibited by iodoacetate and fluoride (inhibitors of glycolysis). Anaerobiosis, cyanide, and dinitrophenol affect only phagocytosis by alveolar macrophages. These cells are,

therefore, apparently dependent on oxidative energy metabolism for phagocytic function. This is a particularly important observation because it reflects a basic difference between alveolar macrophages and other leucocytes. It apparently represents a phenotypic difference and one could easily imagine a genotypic variation based on the knowledge of different metabolic pathways controlled by specific genes.

Further evidence that alveolar macrophages are different from other macrophages comes from the work of Myrvik, and others (1961 a,b), and Leake, Gonzeler-Ojeda, and Myrvik (1964). In 1961, these workers obtained alveolar macrophages and oil-induced peritoneal macrophages from rabbits and examined them quantitatively for lysozyme, acid phosphatase and beta glucuronidase. The extracts from alveolar macrophages contained 2-5 times higher concentrations of the three enzymes than those from oil-induced peritoneal macrophages. For example, the average lysozyme level for alveolar macrophages was approximately 2800 µg/ml packed cells, whereas the peritoneal cells contained about 600 µg/ml packed cells. Pavillard (1963), in a separate study involving rats, supported the observations of Myrvik, et al. He also found that acid phosphatase and lysozyme levels were considerably higher in alveolar macrophages as compared with peritoneal macrophages.

The alveolar macrophage is at least quantitatively different in enzymatic content from other macrophages. It

also seems to engage in oxygen dependent metabolism, whereas peritoneal monocytes may be facultative with respect to oxygen requirements. A question then arises as to the origin of alveolar macrophages. It is important to know whether phagocytes with the characteristics mentioned before arise from the lung tissues and cells or whether they come from the peripheral blood, acquiring their special nature once they arrive. The answers to these questions are still being sought. Many of the early investigators were convinced that alveolar macrophages originate from the lung itself (Leake, Gonzeler-Ojeda, and Myrvik, 1964). Textbooks of histology usually either imply that alveolar macrophages can come from the lung or they provide inconclusive information. More recently, some investigators have considered both theories of origin (Cohn, 1968). Pinkett, Cowdrey, and Nowell (1966), using karyotypic markers, found that at least 50 per cent of the alveolar macrophages come from the peripheral circulation. In general, the use of karotypes for the study of mononuclear phagocytic kinetics has the deficiency of not allowing for examination of the morphology of the cell in detail and of depending on the mitotic activity in situ, which in many instances is quite low.

Normally, inhaled bacteria disappear rapidly from the lungs of experimental animals. This early clearance of bacteria is thought to be an important process in non-specific resistance to infection in the bronchopulmonary tree. A

wide variety of chemical, hormonal, and environmental agents depress, to varying degrees, the rate at which inhaled viable bacteria disappear from the lungs. Such studies support epidemiologic evidence that multiple agents may be involved in the pathogenesis of chronic infections of the lungs.

It is not clear, however, to what extent each of the several components of the bronchopulmonary tree participates in the initial inactivation of inhaled bacteria. Although the mucociliary stream is frequently credited with this cleansing action, much evidence suggests that bacterial clearance may be accomplished by alveolar macrophages.

In light of the higher enzymatic activity in alveolar macrophages (particularly lysozyme), one might assume that they would be able to dispose of microorganisms effectively. Pavillard (1963), however, presented data showing that alveolar macrophages are less able to kill Escherichia coli and Staphylococcus aureus in vitro than are peritoneal macrophages which presumably have lower levels of enzymes to deal with these bacteria. It is important to remember, however, that it is still not known whether lysosomal enzymes play a role in the bactericidal activity of macrophages. If they do not, then one would have to attach more importance to the mucociliary stream. Yet, studies by Green and Kass (1964) and Green (1968) again point to the lung macrophage as the major factor of early resistance to bacterial infection. When mice were exposed to an aerosol

of ^{32}P-tagged S. aureus and Proteus mirabilis and the rate of decline of bacterial viability was compared with the rate of their mechanical removal, it was found that bacterial viability declined 80 to 90 per cent in 4 hours, whereas radioactivity declined by only 14 to 20 per cent. The marked disparity in these rates indicated to these workers that mechanical removal comprised a relatively small fraction of the total clearing process. They also stated that "the in situ bactericidal action of the lung predominated over the mechanical removal process in achieving clearance of the inhaled bacteria." Another interesting observation relating to bactericidal activity of alveolar macrophages comes from the work of Leake and Myrvik (1964). They found that subcellular particles of rabbit alveolar macrophages containing lysozyme, cathepsin, and acid phospatase were resistant to osmotic shock and that lysozyme was released only when electrolyte (0.15 M NaCl) was added to the system. This is in contrast to the particles of peritoneal macrophages which were very susceptible to osmotic shock. These findings could lead one to the conclusion that the alveolar macrophages do not release their lysosomal contents readily. This conclusion would tend to support the results of Pavillard (1963), but not those of Green and Kass (1964, 1968). This seems rather confusing, however one must remember that there is a difference between clearance and intracellular death. It is quite possible that the majority of organisms phagocytized by alveolar macro-

phages are simply transported via the phagocytes to the mucociliary stream and washed out in expelled air. If one considers the diverse virulence factors of bacteria, it is understandable why a given organism may be killed by phagocytic cells and others are not. In other words, it is to be expected that the ability of phagocytes to kill organisms depends on factors quite distinct from their ability to transport particulate material mechanically to the mucociliary stream. Both factors are important, however, since the pathogenesis of infectious disease depends, in general, on the presence of viable organisms.

It now seems appropriate to review some of what is known about intracellular killing of phagocytized microbes. The mechanisms involved in intracellular killing of phagocytized microorganisms have been the subject of a considerable amount of investigation.

Ever since Metchnikoff's original work demonstrating phagocytosis by certain leucocytes, man has sought an explanation for the antibacterial nature of these body components. Most common bacteria engulfed by neutrophils are rapidly killed. A few microorganisms, such as tubercle bacilli, brucellae, and Histoplasma capsulatum, are not susceptible to killing by neutrophils. Death of microorganisms soon after their engulfment by neutrophils has been established by many different techniques, perhaps the most direct and elegant being those of Wilson, Wiley, and Bruno (1957).

Mixtures of leucocytes and streptococci were observed and photographed under phase contrast microscopy in thin cover slip preparations. At various time intervals following phagocytosis, neutrophils were disrupted, without damage to the bacteria, by passing an electric current through the preparations. Observations were continued for several hours to determine if streptococci liberated from leucocytes were capable of growth. Streptococci which had resided inside the neutrophils for less than five minutes almost always survived and grew, whereas those which had been within neutrophils for 15 minutes or longer were usually dead.

Of the various intraphagocytic substances which might be involved in bactericidal activity, ordinary digestive enzymes such as proteinases, cannot be held entirely responsible, since bacteria generally thrive in the presence of these materials (Salton, 1953). Numerous antimicrobial substances such as peroxide, leukins, and phagocytin have been found in neutrophils and warrant further consideration, even though it is not yet established whether they are active <u>in vivo</u>.

Phagocytic neutrophils produce large quantities of lactic acid in the course of their glycolytic metabolism. Studies employing indicator vital dyes, or the introduction of micro-electrodes, indicated that the pH surrounding engulfed particles may be as low as 3.0 to 5.0 (Cohn and Morse, 1960). This degree of acidity, especially in the

presence of organic acids, could be lethal to many types of microorganisms. Acidity of the phagocytic vacuole, like the acidity of the stomach juices in man, may exert an antimicrobial effect. Acid alone cannot, however, account completely for microbial death in neutrophils. Many bacteria which can survive at acid pH, for example certain coliforms, are rapidly killed in these cells.

Lysozyme, an aminopolysaccharidase discovered by Fleming and Allison (1922), is present in large amounts in neutrophils (approximately 2.0 mg of lysozyme per gram wet weight of packed cells) and, furthermore, it is associated with the cytoplasmic granules. Lysozyme degrades the cell wall and causes lysis of certain species of bacteria and, in conjunction with other agents such as acid, cation chelators or antibody and complement (Tagashira, et al., 1953), kills other types of microorganisms as well. Whether lysozyme behaves in neutrophils primarily as an antibacterial agent or as a digestive enzyme remains to be established. Perhaps both activities are operative, at least in some situations.

Antimicrobial basic proteins and peptides, usually called "leukins," have been extracted from neutrophils (Skarnes and Watson, 1956). Their suppressive effect on microorganisms is not disputed, but reasonable doubt exists as to their occurrence in the living cell at sites accessible to contact with ingested bacteria.

Hirsch (1956) has extracted from neutrophils of various animal species a bactericidal substance called "phagocytin." This material has never been purified, but its behavior is consistent with that of a basic protein. Very low concentrations, less than 1.0 µg/ml of the crude phagocytin preparations, promptly kill a wide range of microorganisms without lysing them; the mechanism of its action is not known.

Until about 1965, it was known only that degranulation of polymorphonuclear leucocytes occurred during or after phagocytosis. Because of the work of Hirsch and Cohn (1960) and Hirsch (1962), it is known that bacteria ingested by neutrophils do not lie free in the cytoplasm but, rather, are contained in a pouch formed by cell membrane invagination during phagocytosis. Similarly, digestive enzymes and antimicrobial substances of the neutrophils are not dissolved in the cytoplasm, but are confined within membrane bound granules referred to as "lysosomes." When the membrane of the granules and that surrounding the ingested particle come into contact, they apparently fuse with resulting discharge of lysosomal contents directly into the phagocytic vesicle. The resulting structure has been called the "phagolysosome" by de Duve and Wattiaux (1966). Thus, neutrophil engulfment and digestion are similar in many respects to that in higher multicellular organisms (man and animals); the cell ingests particulate matter (food) in a vesicle rather than into the

surrounding cytoplasm. This mechanism provides a means for controlled granule lysis and, furthermore, probably explains how the neutrophil can degrade engulfed material without, at the same time, digesting its own cytoplasm and nuclear structures.

Even though many facts concerning phagocytosis at the cellular and subcellular level have been uncovered, the specific mechanism causing intraphagocytic death of ingested organisms is still unknown. A step toward understanding this mechanism has come to light slowly during the last two decades. An enzyme, isolated and purified from subcellular components, has been discovered which has potent antimicrobial properties. The enzyme has been called "myeloperoxidase." Originally, "verdoperoxidase" was isolated and partially purified by Agner (1941). Shortly thereafter, the name was changed to myeloperoxidase because investigators were able to extract large quantities of it from leucocytes of patients with myeloid leukemia. The importance of the enzyme was probably not realized until Klebanoff and Luebke's work was published in 1965 (Klebanoff and Luebke, 1965). These workers were investigating the antibacterial properties of bovine saliva and found the peroxidase activity to be high. Also, they found a dialyzable component which was identified as a thiocyanate ion. Hydrogen peroxide was also noted to be present in significant amounts. When the three components (salivary peroxidase, thiocyanate ion and H_2O_2)

were combined *in vitro*, they effectively killed *Lactobacillus acidophilus*. The authors also found that bovine milk had large quantities of lactoperoxidase. Morrison and Allen (1963), a short time before, had shown that lactoperoxidase from bovine milk was immunologically indistinguishable from bovine salivary peroxidase.

At about the same time, Reiter, Pickering, Oram and Pope (1963) showed that the antimicrobial system of milk requires thiocyanate ions in addition to lactoperoxidase, and they suggested that the milk and saliva antimicrobial systems may be similar. In the studies mentioned previously, hydrogen peroxide was not incorporated, however catalase added to the antimicrobial system prevented inhibition of growth of *Lactobacillus acidophilus*. This was *prima facia* evidence for the conversion of H_2O_2 to O_2 and water. Realizing the foregoing facts, Klebanoff, Clem and Luebke (1966) tested the antimicrobial properties of the lactoperoxidase-thiocyanate-H_2O_2 system. He was able to show killing of various microorganisms under test conditions. Moreover, myeloperoxidase from canine PMN's could substitute for the lactoperoxidase of bovine milk. When catalase was added to the system, microbial killing was prevented, indicating that hydrogen peroxide was an important component. Later, it was shown that several halides, in the proper concentration, could substitute for the thiocyanate ion (Klebanoff, 1967 a; Klebanoff, 1967 b).

Agner (1941) estimated the peroxidase content of human myeloid leukemic leucocytes to be about 1-2 per cent of the dry weight of these cells, whereas Schultz and Kaminker (1962) suggested that the normal human neutrophil has a peroxidase content of greater than 5 per cent of the dry weight. In 1967, Klebanoff (1967 b) undertook another study of the bactericidal activity of myeloperoxidase (MPO). This time he replaced the thiocyanate ion with iodide in the MPO system. *In vitro* he noted that the MPO-iodide-H_2O_2 system was effective in killing *E. coli* and that the individual components lacked bactericidal activity.

Iodide ions are readily oxidized by peroxidase and H_2O_2 to iodine, a known antimicrobial agent (Klebanoff, Yip, and Kessler, 1962; Taurog and Gamble, 1966). The first assumption of the former authors was that the antibacterial effect of the system was due to the accumulation of iodine in the reaction mixture. Thirty minutes of preincubation of MPO system components without the addition of *E. coli* caused production of iodine. When *E. coli* was added to the preincubated products, no killing of the organisms was observed. Clearly, the organism must be present in the MPO reaction mixture for the peroxidase system to be effective.

Apparently, myeloperoxidase catalyzes the formation the iodine-carbon bonds when incubated with iodide ion, hydrogen peroxide and a suitable iodine acceptor such as tyrosine or tyrosine residues of proteins (Klebanoff, Yip,

and Kessler, 1962; Taurog and Gamble, 1966). A characteristic of the iodination reaction catalyzed by peroxidase is that the iodine acceptor must be present in the reaction mixture during iodide oxidation for optimum iodination. The similarity to the bactericidal effect of myeloperoxidase-iodide-H_2O_2 in this regard is striking.

Now that it is known that the MPO-system operates *in vitro*, one must ask if the system can be applied *in vivo*. First, are the concentrations of the substances required for the MPO-system to operate present *in vivo*? It has already been shown that myeloperoxidase is present in high concentrations within neutrophils (Agner, 1941; Schultz and Kaminker, 1962). Moreover, histochemical studies suggest its localization in the cytoplasmic granules of the cell (Kaplow, 1965). As mentioned before, the lysosomal granules of the neutrophil rupture after phagocytosis and discharge of their contents into the phagocytic vacuole containing ingested microorganisms or particles. This occurs both *in vitro* and *in vivo* (Hirsch and Cohn, 1960; Hirsch, 1962).

Iodide is present in very low concentration in plasma (0.1-0.6 µg/100 ml plasma) (Todd-Sanford, 1969). However, the uptake of iodide by normal human leucocytes has been reported to be 1200 times the uptake by red blood cells (Siegel and Sachs, 1964). Thyroxine and triiodothyronine also are preferentially taken up by leucocytes and the iodination of the hormones by leucocyte preparations has been reported by

Kurland, Krotkov, and Freedberg (1960).

It has been reported that H_2O_2 is formed in the PMN during the period of accelerated metabolism following phagocytosis (Iyer, Islam, and Quastel, 1961; Karnovsky, 1962). Recently, the alveolar macrophage has been noted also to have an increased production of H_2O_2 as a secondary event to particle entry (Bernard, Gee, Vassallo, Bell, Kaskin, Basford, and Field, 1970).

Apparently, all the components for the intracellular in vivo MPO-system are found within the neutrophil, at least in the PMN. Klebanoff and Luebke (1965) have shown that for a bactericidal reaction to occur within the MPO-system the pH must be around 5.0. The intracellular pH in the vicinity of the ingested particle has been reported to be distinctly acid, i.e., in the vicinity of the pH optimum for the iodination reaction. The low pH is, presumably, due to the formation of lactic acid (Karnovsky, 1962).

Even though there is good evidence that iodide may be the significant halide ion active in vivo, there remains some doubt. Accordingly, Klebanoff (1968) sought other halide ions that might be more readily available to the PMN in vivo. Essentially, Klebanoff found that bromide and chloride, but not fluoride could be substituted for iodide in the MPO-system. It should be noted, however, that the halides are not equally effective. In a controlled system containing 5×10^6 E. coli/ml, acetate buffer (pH 5.0), and

150 o-dianisidine units, it required 1.0 μM of chloride and 0.1 μM of bromide to kill all the E. coli, whereas it required only 0.01 μM of iodide to kill the same number of organisms.

In general, however, Klebanoff (1968) found that the properties of the myeloperoxidase-bromide-H_2O_2 or chloride-H_2O_2, bactericidal system was similar to those described previously for the iodide containing system. The deletion of any component (MPO, H_2O_2, bromide or chloride ions) from the reaction mixtures greatly decreased the bactericidal effect. He also noted that preheating the myeloperoxidase for 10 minutes at 90° destroyed its bactericidal effect.

The finding that chloride ions could be substituted for iodide was of great interest. Chloride ions are readily taken up by leucocytes. The average intracellular chloride concentration of rabbit peritoneal lavage leucocytes is 93 meq/kg. of cell water as compared to normal rabbit serum concentration of 115 meq/liter of serum water (Wilson and Manery, 1949). Klebanoff (1968) reported that a bactericidal effect was observed in the MPO-system at a chloride ion concentration of 0.005 meq/liter. This appears to be well within the concentration limits reported to be present in leucocytes.

Presently, most workers believe that the MPO-system is indeed a significant bactericidal mechanism asscciated with phagocytes and phagocytosis. Yet, the picture remains unclear mainly because no one is sure just how the MPO-system

effects killing. Moreover, there is at present a major controversy over which halide ion actually takes part in the MPO-system. As in the past, it remains difficult to compare results of different investigators because different methods were employed. More recent work concerning the biochemical mechanisms of the bactericidal activities of phagocytosis will be presented in the Discussion.

The purpose of this research was to examine the events pertaining to the phagocytosis of C. neoformans by alveolar macrophages. This included such events as intracellular fate, resistance to phagocytosis and mechanisms involved in intracellular degradation or dispersal.

CHAPTER II

MATERIALS AND METHODS

Organisms

Both a non-encapsulated and an encapsulated strain of *Cryptococcus neoformans* (strain CIA) were used in these studies. The following fungi were also utilized in connection with various experiments undertaken in the study: *Sporotrichum schenckii*, *Candida albicans*, *Aspergillus niger*, and *Rhizopus oryzae*. All of the fungi were obtained from the stock collection in the Department of Microbiology and Immunology, University of Oklahoma Health Sciences Center, Oklahoma City, Oklahoma. Three bacteria, *Escherichia coli*, (ATCC 11775), *Salmonella typhosa* and *Seratia marcescens*, were also used in this study.

Animals

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

Media

Non-encapsulated cells of *Cryptococcus neoformans* were grown on a synthetic low pH medium (LpH). This medium was

prepared as follows: Essential salt solution (ESS) consisted of 100 ml of distilled water containing KH_2PO_4, 2.0 gm; $CaCl_2 \cdot 2H_2O$, 0.002 gm; $(NH_4)_2SO_4$, 2.0 gm; $MgCl \cdot 6H_2O$, 0.2 gm; $FeCl_2 \cdot 4H_2O$, 0.04 gm; $MnCl_2 \cdot 4H_2O$, 0.0015 gm; and $NaNO_3$, 0.0015 gm. LpH agar medium was prepared by adding 0.02 gm/ml glucose and 5.0 µg/ml thiamine monochloride to a 1:10 dilution of ESS and 2.0 per cent agar (Difco). Hydrochloric acid (0.1N) was used to adjust the pH to 4.5-5.0.

Growth Media for Aerosolization and ^{32}P Incorporation

Bacto-Yeast Nitrogen Base without Amino Acids (Difco) was used in the first experiments. The pH was adjusted 5.0 with 0.1N HCl.

In an attempt to find a liquid medium that would produce C. neoformans with desirable characteristics as to cell size and abundance of growth, variations of Basic Czapek (Difco) medium were utilized. Modifications included the additions of varying amounts of glucose, $NaNO_3$ and K_2HPO_4, the latter as a source of extrinsic phosphate ions. Czapek (Difco) medium contains: $MgSO_4$, 0.5 gm/1000 ml; KCl, 0.5 gm/1000 ml; $FeSO_4$, 0.01 gm/1000 ml; peptone, 5.0 gm/1000 ml; yeast extract, 1.0 gm/1000 ml. In later experiments, Czapek medium was prepared without peptone and yeast extract. Peptone and yeast extract and/or glucose were added according to the growth and size of the C. neoformans. Also, K_2HPO_4

(range, 0-150 mg/100 ml) were added to Czapek medium in an effort to control size and growth of the organisms. All of these solutions were adjusted to pH 5.0 with 0.1N HCl.

Radioactive Labeled Organisms. Depending on the particular experiment, two 250 ml flasks were inoculated with 2.0×10^6 nonencapsulated C. neoformans which had been starved for 48.0 hours in a pH 7.0 tris buffer. One or two microcuries of $H_2{}^{32}PO_4$ in 0.02N HCl was added to each flask of modified Czapek medium and organisms. The cultures were placed on a mechanical shaker and incubated at room temperature. After 5 days incubation the organisms were harvested by centrifugation and washed 4 times in a pH 5.0 acetate buffer. Next, the washed, radioactive organisms were resuspended in one of the aforementioned media and counted microscopically using a hemacytometer. Samples of the organisms were dilution plated to determine the viability.

Alveolar Macrophages

Guinea pig alveolar macrophages were harvested by a modification of the method of Myrvik, Leake and Fariss (1961 b).

Under aseptic conditions, a midline incision was made in the thoracic area which extended from the cephalic part of the trachea downward to the xiphoid process. Next, the trachea was dissected out and clamped. This caused death of the animal and prevented backwash of normal microbial flora

from the posterior pharynx. Quickly, the intact lungs and trachea were removed from the thoracic cavity and washed vigorously with sterile 0.85 per cent NaCl. Next, the lungs were filled, via the trachea, with cold, sterile Eagle's balanced salt solution (BSS). The trachea was again clamped and the fluid-filled lungs were gently massaged, under sterile conditions, for about 2 minutes. The clamp on the trachea was removed and the Eagle's solution containing the alveolar macrophages was allowed to flow, by gravity, into 15 ml sterile, siliconized centrifuge tubes. The first washing from the lungs was discarded because it contained predominantly the so-called "dust cells" (alveolar macrophages that are metabolically in death phase and containing dust particles), and other cellular debris. The cells obtained from subsequent washings were washed 3 times in Eagle's BSS at a centrifuge speed not exceeding 400 x g. After the final centrifugation, the alveolar macrophages cells were resuspended in cold Eagle's BSS, counted and stained with 1 per cent trypan blue to determine viability.

In Vitro Phagocytosis and Fate

In order to obtain maximum percentage phagocytosis *in vitro* with alveolar macrophages it was necessary to study various physical and biological conditions of the system.

To determine if autologous serum had an influence on phagocytosis, several experiments were tried in which the

serum concentration was varied from 0-65 per cent. At the same time, it was necessary to determine the optimal ratio of yeast cells (C. neoformans-strain CIA) to alveolar macrophages. LpH cells (nonencapsulated) were incubated in 100 x 13 mm siliconized test tubes with the guinea pig alveolar macrophages at 37° C on a rotator (30 rpm) for 2 hours. Samples were removed at 15, 30, 60 and 120 minutes incubation and scored for percentage phagocytosis by microscopic examination of stained cells. This phagocytic procedure was employed for each experiment where there was a variation in the yeast cell ratio and the percentage serum. All ratio experiments consisted of an average of 5 separate determinations. Thirty per cent autologous serum was used in the ratio experiments. One hundred alveolar macrophages were counted and the number of alveolar macrophages containing cryptococcal cells was recorded as the percentage phagocytosis. The phagocytic index was determined by enumerating the average number of yeast cells ingested per 100 alveolar macrophages.

In some instances, purified capsular material was added to the system to see if it had any effect on phagocytosis by the alveolar macrophages.

In vitro intracellular fate experiments were performed after the optimal conditions of phagocytosis had been determined. Fate experiments were similar to the phagocytosis procedure except for certain experimental modifica-

tions. The individual cryptococcus-serum suspensions were dispensed, 0.5 ml per tube (6 x 10^6 cells/ml), into two sets of siliconized test tubes (100 x 15 mm). To each tube in set 1, 0.5 ml of freshly collected alveolar macrophages was added (2.0 x 10^6 alveolar macrophages/ml), making the ratio of alveolar macrophages to yeast cells in the fate experiments about 1:3. In set 2, 0.5 ml of sterile 0.8 per cent NaCl was added instead of the alveolar macrophages suspension. In set 3, 0.5 ml of the alveolar macrophages suspension was added, followed by 0.5 ml of 0.5 per cent sodium deoxycholate (DOC) to disrupt the alveolar macrophages.

Tubes from sets 1, 2, and 3 were incubated at 37° C on a rotor (Scientific Industries, Inc.) at 30 rpm. At 0, 2 and 4 hour intervals, 0.5 ml of 0.5 per cent DOC was added to sets 1 and 2 and viable plate counts, using Sabouraud Dextrose Agar (Difco) medium, were made from all three sets to enumerate the number of viable cells of C. neoformans.

The same procedures of phagocytosis and fate were employed for studies on human alveolar macrophages except that larger volumes of Eagle's BSS were used to lavage the human lungs. The human lungs which provided alveolar macrophages were obtained from a patient who died from a cerebral vascular accident. The human alveolar macrophages were no more than 5 hours old when used.

On several occasions, to obviate the possibility of poor alveolar macrophages viability and function, E. coli

(ATCC 11775) was utilized in fate experiements. The conditions employed were the same except that the number of E. coli utilized was 1 x 10^6/ml in 40 per cent guinea pig serum and normal saline (0.85 per cent NaCl).

Electron Microscopy

In an attempt to visualize the guinea pig alveolar macrophages, electron microscopy was employed. Freshly isolated guinea pig alveolar macrophages were centrifuged at 1200 x g to form a pellet. The pellet was fixed in 4 per cent cacodylate buffered glutaraldehyde for 35 minutes, after mixing well. The alveolar macrophages were recentrifuged and resuspended in physiological sucrose buffer for 20 minutes. After recentrifugation for pellet formation, each sample was post-fixed in an osmium fixative for 20 minutes, then washed and resuspended in veronal buffer. Next, each sample was dehydrated in graded alcohols: 2 changes of 70 per cent for 10 minutes each, 2 changes of 95 per cent for 15 minutes, and 2 changes of 100 per cent ethyl alcohol for 20 minutes. Each sample was then resuspended in propylene oxide, 2 changes for 10 minutes each. Next, the samples were placed in a 50:50 mixture of propylene oxide and Epon 812 for 1.0 hour. After this, the samples were removed from the 50:50 mixture and allowed to remain in pure Epon 812 for 1.0 hour. Next, the samples were divided into two parts and put into gelatin capsules. One group was embedded in Epon 812 and the other

group was back-up embedded in Araldite. The samples were allowed to polymerize at 60° C overnight before sectioning. Sectioning was performed on a Sorvall Porter-Blum ultramicrotome, Model MT-1. Glass knives were used which had been 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. Voltage employed during observation was 100,000 kv.

In Vivo Fate with Labeled Organisms

In attempts to determine the fate of *C. neoformans* deposited in guinea pig lungs, several experiments were performed.

Aerosolization

First attempts to deposit yeast cells in guinea pig lungs employed a Tri-R Infection Apparatus (Tri-R Instruments, Inc.). This is a self-contained nebulizing unit which produces a cloud containing particles to be inhaled. After controlled nebulization, the particles enter an enclosed, stainless steel tank containing the animals to be infected, which inhale the organisms in a simulated, natural fashion. The infectious cloud can be exhausted through bacteriological filters and the animals' exterior surface can be sterilized by strong ultra-violet radiation. The time intervals and the numbers of particles entering the

chamber can be regulated externally by air flow control mechanisms. Two or three guinea pigs (approximately 700 gm each) were placed in the Tri-R apparatus for each experiment. On some occasions, before aerosolization, each animal received 0.5 ml of 1:1000 adrenalin, intraperitoneally, in an attempt to retard excessive bronchial secretions. Previously $H_2{}^{32}PO_4$-labeled organisms grown in the aforementioned special broth were used as a source of infectious particles to be placed in the nebulizer. Nebulization was carried out for a total of approximately 40-50 minutes (time depended upon formation of an adequate cloud and this depended upon humidity inside and outside the chamber, since the apparatus used filtered, atmospheric air.) Cloud decay and decontamination generally lasted for 40 minutes. Total fluid aerosolized ranged from 27-29 ml of specific Czapek broth containing approximately 9.0×10^5 yeast cells/ml. The mean diameter of the organisms was 5.37 μ (±1.39, S. D.).

Transtracheal Instillation. In this procedure C. neoformans cells, grown in Czapek broth with peptone and yeast extract (P/YE broth), were introduced aseptically into the lungs. The technique involved exposing the trachea surgically and injecting viable yeast cells into the lungs via the trachea. Best results were achieved when the organisms were introduced in a concentrated form ($1.5\text{-}1.7 \times 10^7$ cells) during inspiration.

After both the instillation and the aerosolization procedures, the animals were sacrificed at various time periods up to 6 hours by clamping the trachea. The removal of lungs was accomplished by a technique similar to that described for collection of the alveolar macrophages, except that the animals removed from the Tri-R apparatus were washed in a hot soap solution to remove radioactive particles. Each set of lungs was dissected free of the trachea and main stem bronchi. Next, the lungs were homogenized in a glass homogenizer containing 15 ml of cold P/YE broth or cold Eagle's BSS. Samples of the homogenate were dilution plated onto Sabourauds dextrose agar. Three days later, the plates were examined for C. neoformans colonies which were enumerated. The remaining homogenate was counted on a Nuclear-Chicago liquid scintillation counter (Des Plaines, Illinois). A total of 3 guinea pigs was tested for each time period during the transtracheal instillation experiments.

Activated Alveolar Macrophages and In Vitro Fate

Activated macrophages apparently have a large complement of lysosomal enzymes and, therefore, may be able to kill ingested organisms with greater efficiency (Axline, 1968). In an attempt to activate guinea pig alveolar macrophages, 6 animals were treated as follows: 3 guinea pigs were inoculated intravenously (IV) with 3.0×10^6 heat killed Serratia marcescens and 3 were inoculated with 3.0×10^6 heat killed

Salmonella typhosa. Ten days later, the animals were sacrificed and their lungs were excised to provide a source of activated macrophages. Previously described fate studies were carried out with alveolar macrophages from each animal to determine their ability to kill C. neoformans. Acid phosphatase and beta-glucuronidase levels were also determined and compared with those of normal alveolar macrophages. These enzymes are usually elevated in activated macrophages (Axline, 1968).

Lysosomal Enzyme Extraction

Polymorphonuclear leucocytes (PMN's) from 500 ml of fresh human blood were collected on a leucopac (essentially, a chromatograph column packed with glass wool) at 37° C. Red blood cells and lymphocytes were washed from the leucopac with Eagle's Minimal Essential salts medium (Hyland Labs, La Jolla, California). At this point, the leucopac was either frozen until needed or used immediately. PMN's were removed from the column by washing it with 200 ml of 0.25 M sucrose to which 4000 units of sodium heparin (Organon, West Orange, New Jersey) had been added. The PMN suspension was concentrated by centrifugation at 27,000 x g for 10 minutes. The pellet of PMN's was resuspended in 7.0 ml of the sucrose and heparin solution. Next, the pellet suspension was mixed for 5 minutes on a LaPine mixer in a test tube with a Tri-R teflon tissue homogenizer. The resulting cell homogenate was centrifuged for 10 minutes at 600 x g to remove nuclei, cell

wall fragments, and glass wool. The supernatant fluid containing the granules was decanted off and held at 4° C for the next step. The pellet was suspended again in sucrose-heparin and centrifuged under the same conditions. The two supernatant fluids were then combined and centrifuged at 27,000 x g for 20 minutes. The supernatant fluid was discarded and the pellet of PMN granules was resuspended in 0.25 M sucrose (without heparin) and lysed by freeze thawing 6 times. This material contained the myeloperoxidase preparations used throughout the research.

Enzyme Activity

Peroxidase acts upon hydrogen peroxide as follows:

$$\text{peroxidase} + H_2O_2 \longrightarrow \text{compound}$$

$$\text{compound} + AH_2 \text{ (donor)} \longrightarrow \text{peroxidase} + H_2O + A$$

The rate of decomposition of H_2O_2 by peroxidase with o-dianisidine as a hydrogen donor is determined by measuring the rate of color development at 460 na.

The stock substrate was prepared as follows: 1.0 ml of 30 per cent H_2O_2 (Merck's Superoxol) was diluted to 100 ml with distilled H_2O. Working substrate was prepared by diluting 1.0 ml of stock H_2O_2 to 100 ml with 0.01 M phosphate buffer, pH 6.0 (prepared fresh daily). The dye, 1 per cent o-dianisidine in methyl alcohol, was also prepared fresh daily. Procedure: 0.05 ml of dye was added to 6.0 ml of working substrate. From this solution, 2.9 ml was trans-

ferred to a glass cuvette and the remainder to a control cuvette. At zero time, 0.1 ml of PMN homogenate was added to the first cuvette. The contents of the cuvette were mixed by inverting and the absorbancy was recorded every 15 seconds (B & L Spectronic 20) for 2.0 minutes to determine the rate of change per minute. One unit of peroxidase activity is that amount of enzyme decomposing 1.0 µm of peroxide per minute at 25° C.

Protein was measured by the Biuret (Gornall, Bardawill, and David, 1949) method and the activity of each enzyme preparation was calculated and adjusted to a standard activity of 150 units/0.1 ml of PMN homogenate.

Alveolar macrophages harvested from guinea pig lungs and human lungs were tested for the presence of myeloperoxidase. The methods for enzyme extraction and assay were the same and the macrophages were collected as previously described.

Assay of Fungicidal Activity

One hundred and fifty units of the myeloperoxidase preparation was added to 2.0 ml of sterile 0.1 M phosphate buffer, pH 5.0, in a siliconized test tube (100 x 15 mm). Appropriate amounts of NaI, NaCl, and living organisms were added. Finally, in order to initiate the reaction, various amounts of H_2O_2 in phosphate buffer were added. Tubes were incubated at room temperature. At 0, 15, 30, 60 and 90

minutes, 1.0 ml was removed from the reaction mixture and diluted in 9.0 ml of sterile distilled H_2O. The serial dilutions were plated on either Sabouraud Dextrose Agar or Brain Heart Infusion Aga and incubated at room temperature until colonies could be visualized with 6x magnification.

In certain experiments, inhibitors of the myeloperoxidase reaction were employed. The following inhibitors were used in varying amounts: 3 amino-triazole and potassium cyanide.

Acid Phosphatase. Lysosomal granules which provided the myeloperoxidase preparations were also tested for acid phosphatase and beta-glucuronidase activity. Enzyme activity was determined according to the methods described in the Worthington Biochemical Enzyme Manual (Worthington, Freehold, New Jersey). Acid phosphatase acts on o-carboxy phenyl phosphate. The rate of hydrolysis of o-carboxy phenyl phosphate is determined by following the increase in absorbancy at 300 mu due to liberation of salicylic acid. One unit is equivalent to one micromole of o-carboxy phenyl phosphate hydrolyxed per minute, at pH 5.0 and 25° C.

Beta-glucuronidase. Detection of beta-glucuronidase was also determined by the methods described in the Worthington Biochemical Enzyme Manual. The enzyme acts on phenolphthalein glucuronidate at the optimum pH of 4.5. One unit of beta-glucuronidase activity is that amount which cleaves one micromole of phenolphthalein glucronide per minute at

37° C.

In both enzyme assays, controls consisted of all reaction components except the myeloperoxidase preparation, which was substituted with distilled H_2O.

CHAPTER III

RESULTS

In Vitro Phagocytosis Studies

Results of initial phagocytosis experiments indicated that guinea pig alveolar macrophages could phagocytize nonencapsulated C. neoformans, strain CIA, within 15 minutes. Based on this observation, it was decided to study the biological conditions necessary to achieve maximum phagocytosis in vitro. Serum promotes engulfment in many in vitro and in vivo situations. Therefore, it was decided to learn what concentration of serum (if any) would provide the maximum phagocytosis of C. neoformans. The effect of various serum concentrations in an in vitro environment are shown in Figure 1. Although the experiments were allowed to proceed for 120 minutes, maximal phagocytosis was achieved in 60 minutes. The control, which was the system without serum, showed only an 18 per cent phagocytosis; the system containing 65 per cent autologous serum showed approximately 48 per cent phagocytosis. The system that employed 45 per cent gave 52 per cent phagocytosis, whereas the 30 per cent serum system gave the highest (57 per cent) phagocytosis. The yeast cell to

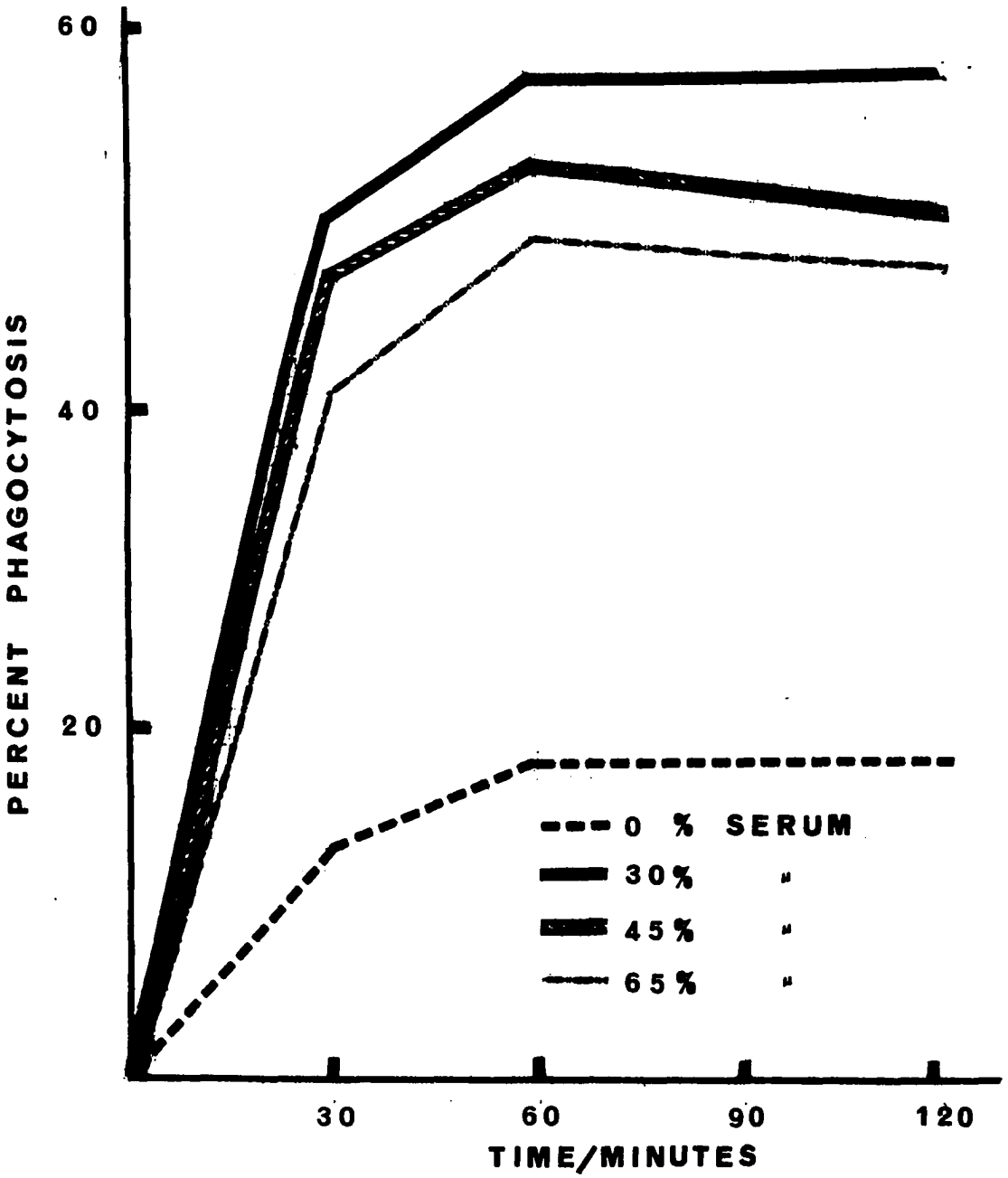

Figure 1---Optimal Phagocytosis Experiment: Effect of serum concentration on phagocytosis by alveolar macrophages.

alveolar macrophage ratio in these experiments was approximately 3:1.

Once the optimal serum concentration was determined, the next step was to vary the yeast cell alveolar macrophages ratio. Figure 2 demonstrates that all the yeast cell to alveolar macrophages ratios, except the 1:1, yielded good percentage phagocytosis. However, when one examines the phagocytic index, it is evident that the 2:1 and 4:1 ratios had better indicies and thus provided the optimal conditions for phagocytosis.

Effect of Capsule Polysaccharide on Phagocytosis

Since cryptococcal polysaccharide is known to inhibit phagocytosis by human PMN's, an experiment was devised to determine if it also inhibits phagocytosis by alveolar macrophages. The usual phagocytic assay was carried out using 30 per cent autologous guinea pig serum, alveolar macrophages and nonencapsulated cryptococcal cells. To this system, varying amounts of partially purified cryptococcal capsular polysaccharide was added. At the end of 60 minutes, the percentage phagocytosis was determined. Table 1 demonstrates these results. It required only 100 µg of capsular material to reduce the percentage phagocytosis from 52 per cent (control) to 30 per cent. Adding up to 1,800 µg of capsular material reduced the percent phagocytosis by only 6 more percentage, i.e., to 24 per cent.

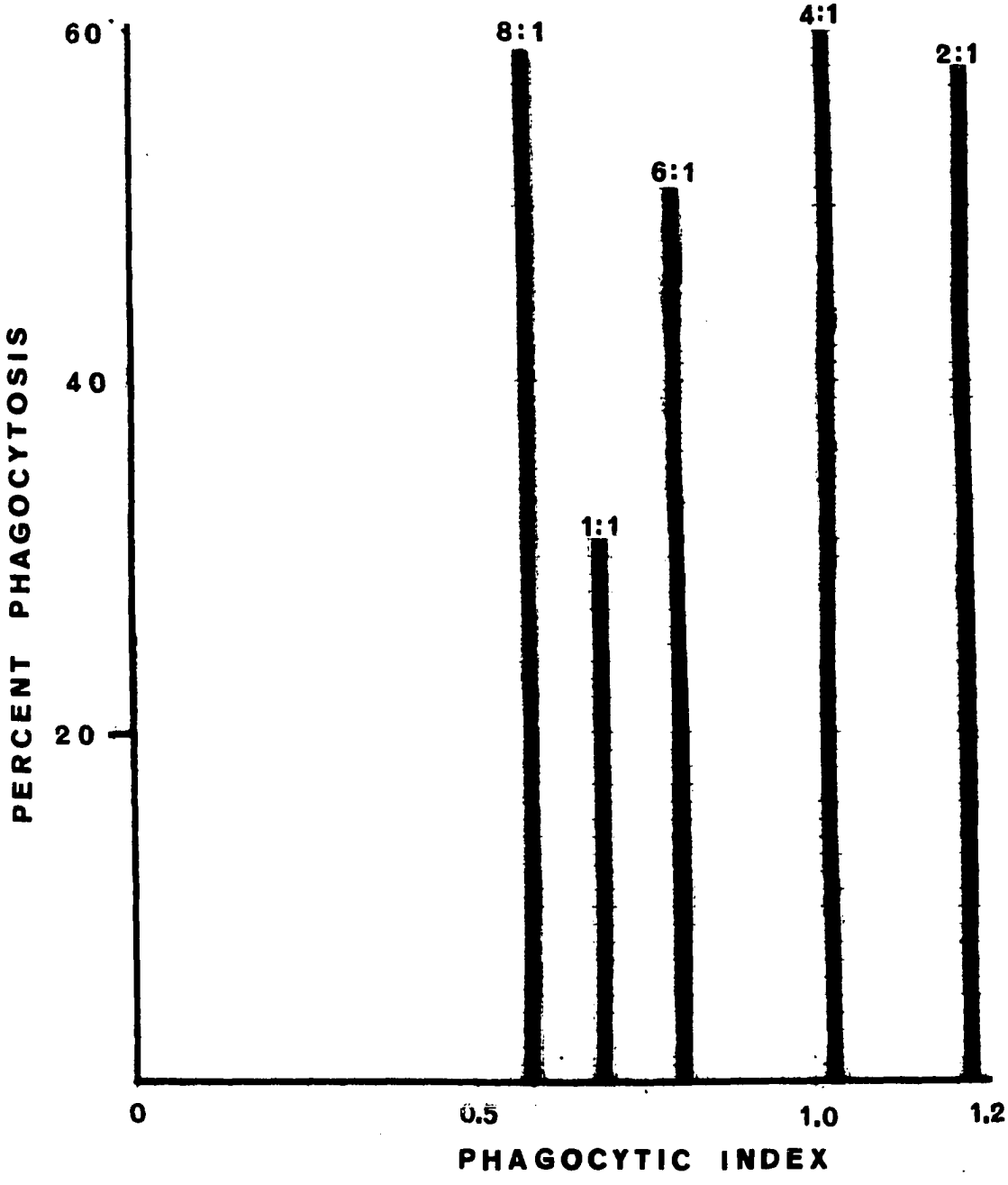

Figure 2--Optimal Phagocytosis Experiment: Effect of yeast cell to alveolar macrophage ratio on percentage phagocytosis and phagocytic index.

TABLE 1

GUINEA PIG ALVEOLAR MACROPHAGES: INHIBITION OF PHAGOCYTOSIS BY C. NEOFORMANS CAPSULAR MATERIAL

Capsule Material µg	Percentage Phagocytosis
1,800	24
1,500	22
1,200	21
900	27
600	26
300	29
100	30
0	52

In Vitro Fate Studies

An important objective of the research was to determine if guinea pig alveolar macrophages could kill the phagocytized cryptococcal cells. Figure 3 is a graphic representation of the results. The results indicate that 33.9 per cent of the cryptococcal cells remained viable in the system with alveolar macrophages (AV/W) at the end of 4 hours, and that 31.3 per cent of the cryptococcal cells remained viable in the system without alveolar macrophages (AV/WO). Trypan blue uptake by the guinea pig alveolar macrophages at the end of the 4 hours incubation showed that 84 per cent of the phagocytic cells remained viable.

To obviate the possibility that the alveolar macrophages were functionally inadequate, Escherichia coli was tested in the phagocytic system. Figure 4 demonstrates that under the same conditions, only 4.6 per cent of the E. coli remained viable after 2.0 hours incubation with the guinea pig alveolar macrophages.

Activated Alveolar Macrophages

It has been shown that macrophages may be activated by endotoxin and by gram negative bacteria. Activated macrophages contain greater quantities of lysosomal enzymes and, in certain instances, have been shown to have increased bactericidal capacity. Heat killed Salmonella typhosa and Serratia marcescens were therefore injected into guinea pigs

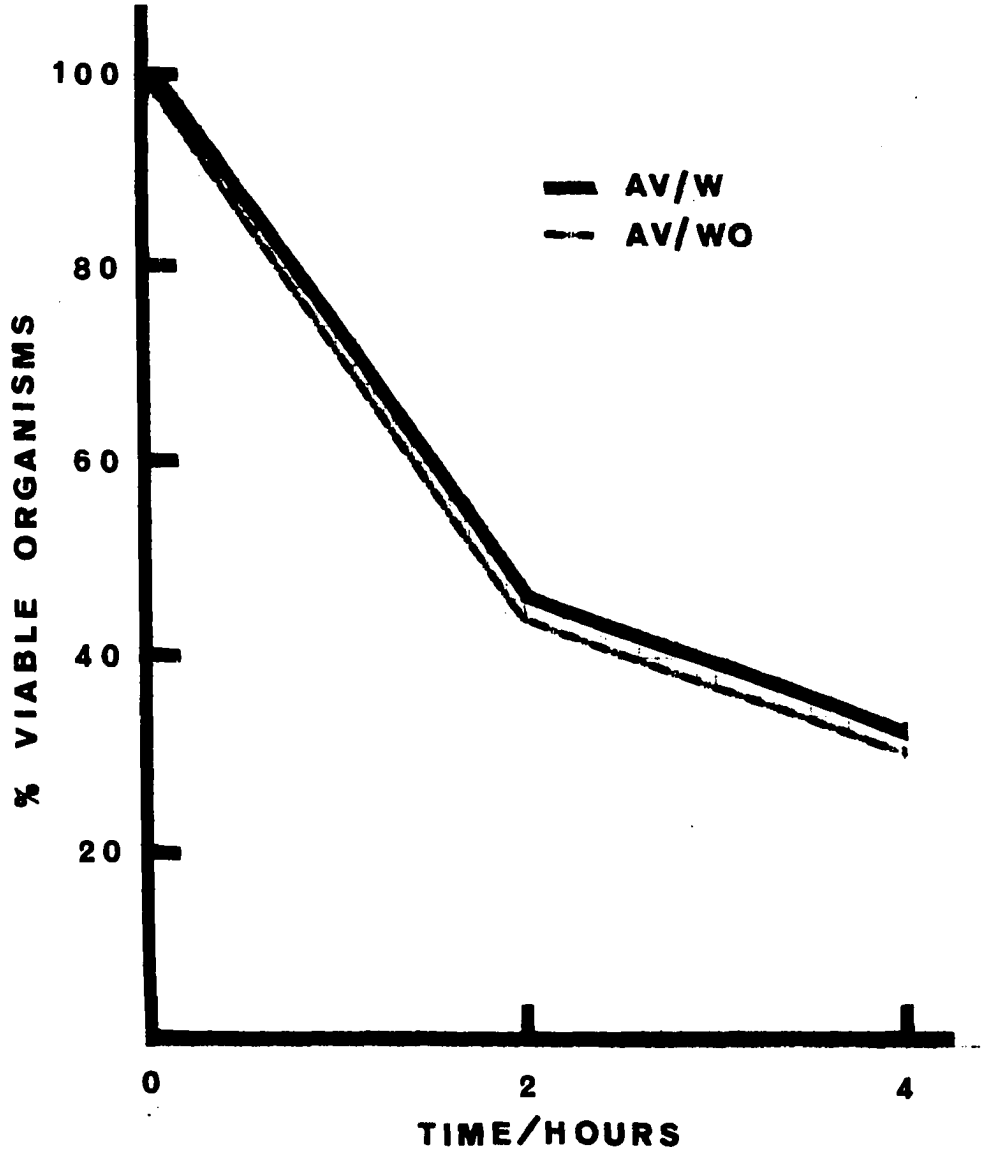

Figure 3--Alveolar Macrophage Fate Experiment: Non-encapsulated C. neoformans incubated with (AV/W) or without (AV/WO) alveolar macrophages.

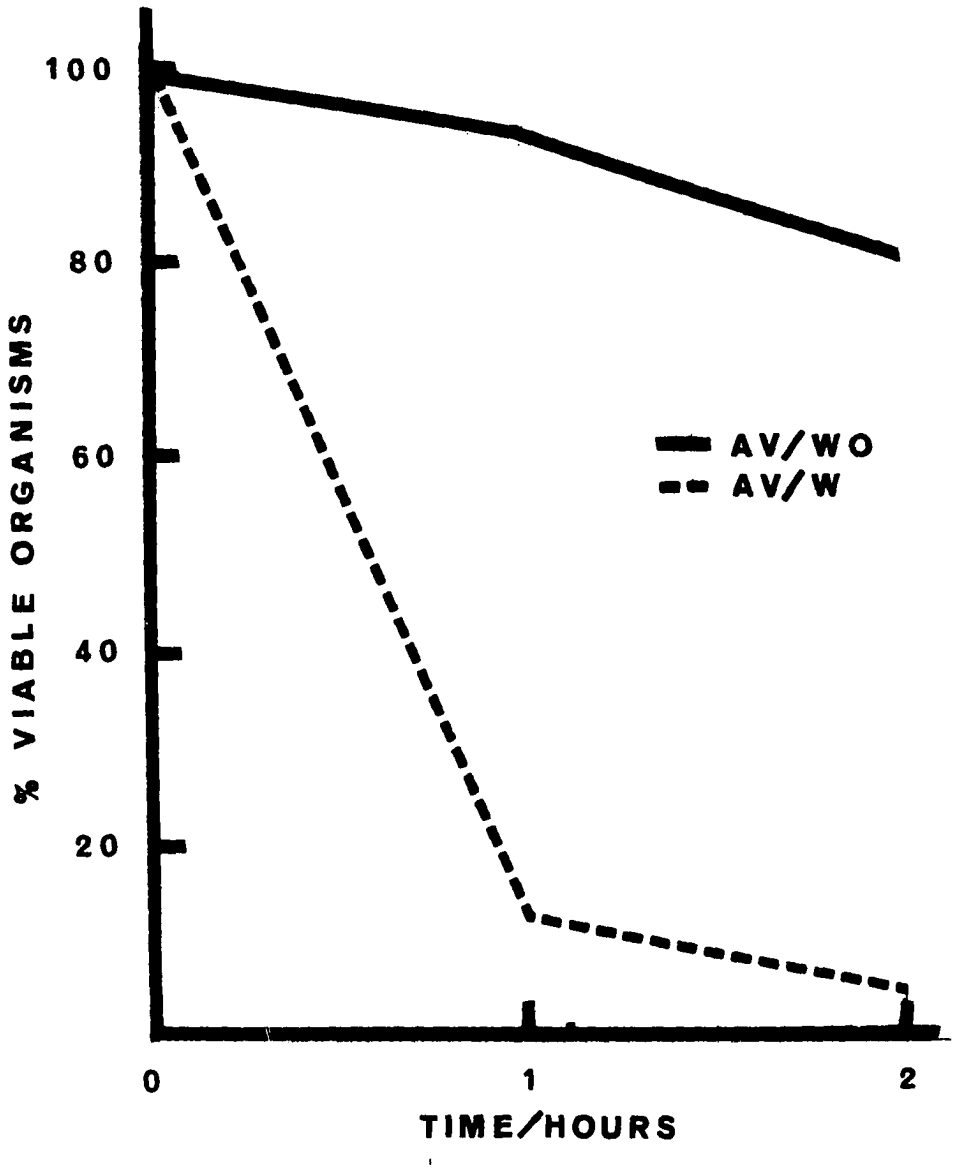

Figure 4--Alveolar Macrophage Fate Experiment: E. coli incubated with (AV/W) or without (AV/WO) guinea pig alveolar macrophages.

in an attempt to activate their alveolar macrophages. Table 2 shows the results of these studies. It is apparent that both organisms caused an increase in enzyme activity (acid phosphatase and beta-glucuronidase) per mg of protein. Acid phosphatase went from an average of 96.0 to 128.7 (activity/mg protein) and beta-glucuronidase went from 27.3 to 33.6 (activity/mg protein).

Activated alveolar macrophages from these animals were tested for their ability to kill non-encapsulated cryptococcal cells. Figures 5 and 6 show the results of fate experiments using S. typhosa and S. marcescens activated macrophages, respectively. Note that after 4.0 hours incubation, no difference in killing was observed in the presence or absence of activated alveolar macrophages.

Phagocytosis by Human Alveolar Macrophages

One of the long range goals of this research was to determine if human alveolar macrophages play a role in the pathogenesis of cryptococcosis. An opportunity arose to possibly answer this question when a piece of human lung became available from a patient who died from a cerebral vascular accident.

The right lower lobe of the lung was lavaged with Eagle's BSS and the alveolar macrophages were harvested. The alveolar macrophages were used in a standard phagocytic system in an attempt to see if they could phagocytize non-

TABLE 2

ACID PHOSPHATASE AND BETA-GLUCURONIDASE ACTIVITY
OF NORMAL AND ACTIVATED ALVEOLAR MACROPHAGES

Enzyme	Normal alveolar macrophages	*S. typhosa* activated alveolar macrophages	*S. marcescens* activated alveolar macrophages
	Active/mg protein	Active/mg protein	Active/mg protein
Acid Phosphatase	96.0	128.7	119.3
Beta-glucuronidase	27.3	33.6	37.2

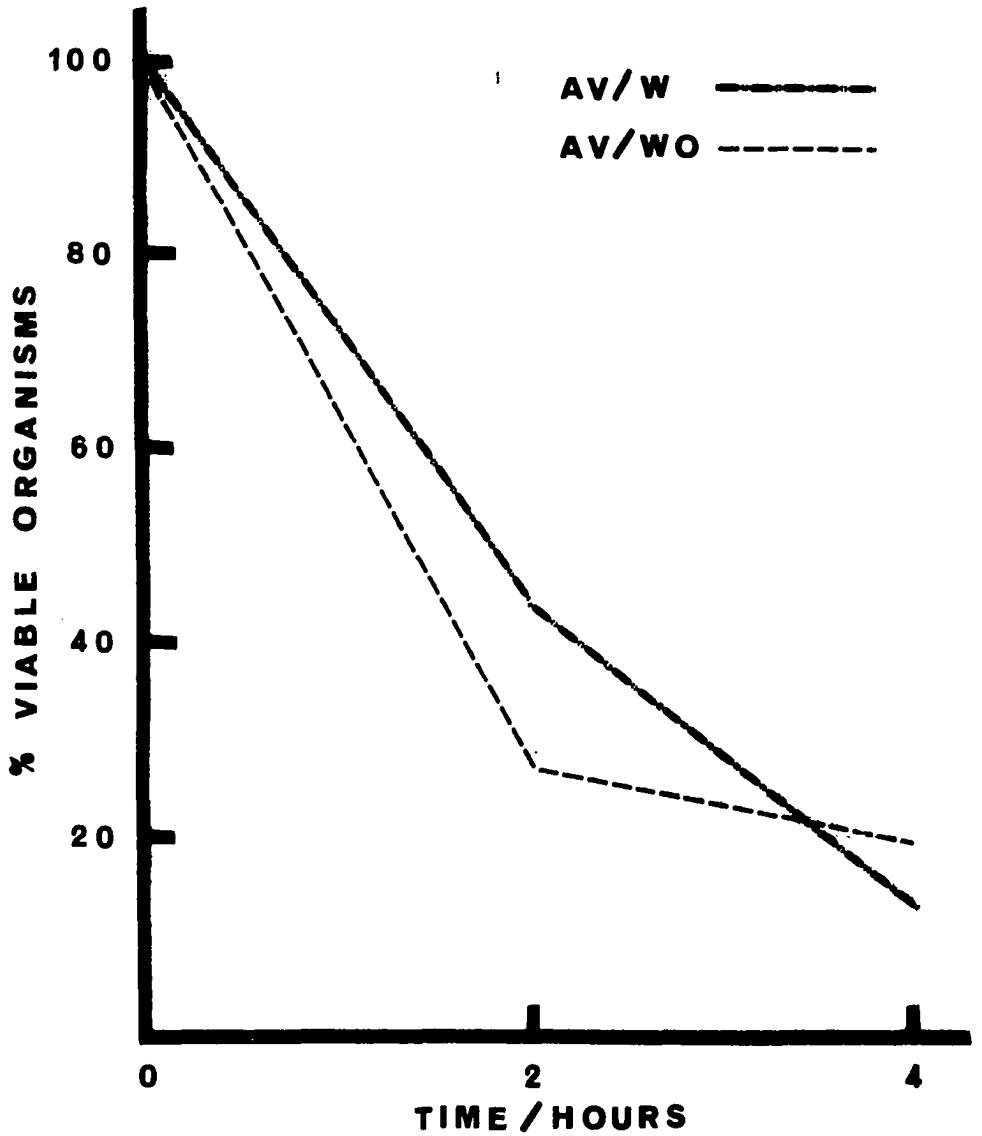

Figure 5--Activated Macrophage Fate Experiment: Non-encapsulated C. neoformans incubated with (AV/W) or without (AV/WO) guinea pig alveolar macrophages activated with heat killed Salmonella typhosa.

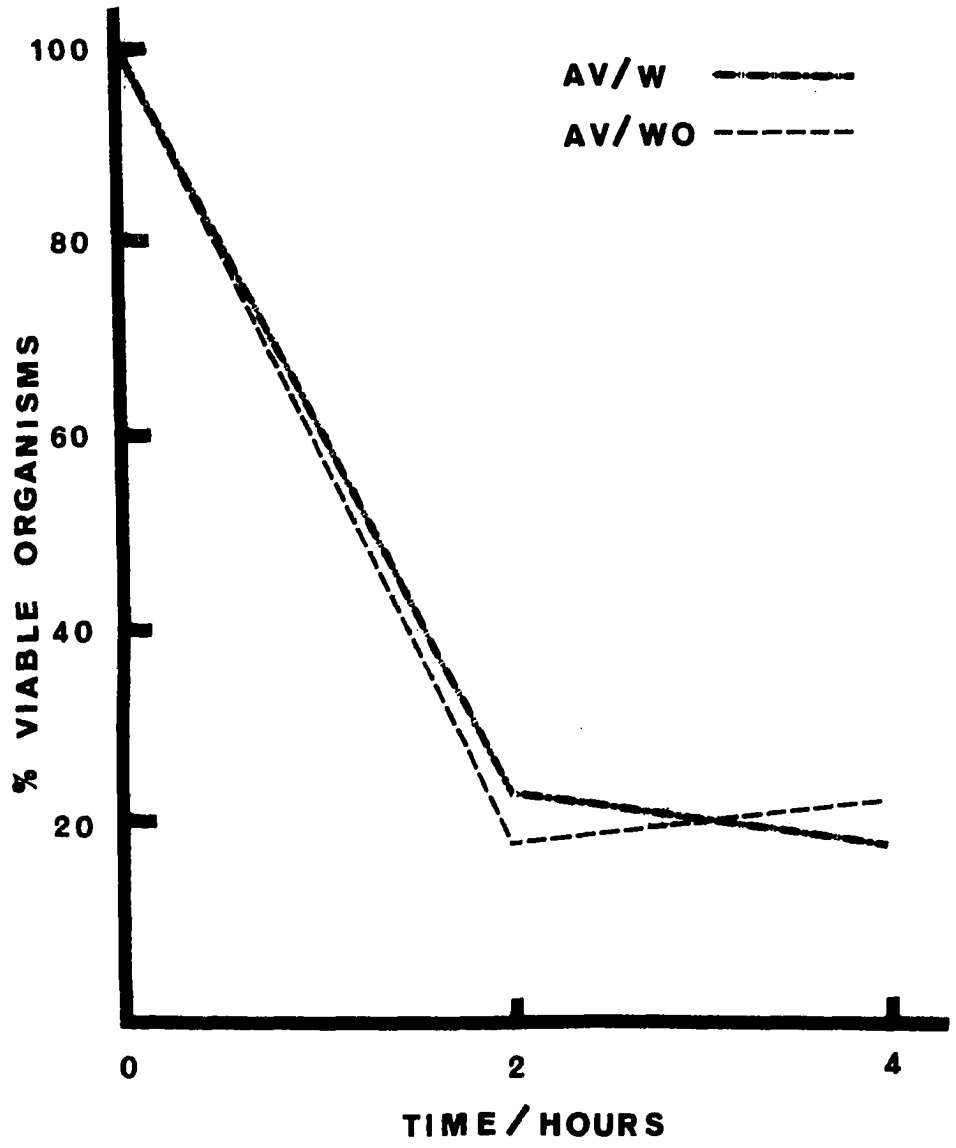

Figure 6--Activated Macrophage Fate Experiment: Non-encapsulated C. neoformans incubated with (AV/W) or without (AV/WO) guinea pig alveolar macrophages activated with heat killed Serratia marcescens.

encapsulated cryptococcal cells. After 60 minutes incubation at 37° C, samples of the cells were observed for percentage phagocytosis. Essentially no phagocytosis (2.0 per cent) was observed. Examination of the alveolar macrophages by trypan blue indicated that less than 40 per cent of the cells were viable. Light microscopic observation revealed a lack of cellular detail and poor morphology.

Electron Microscopy of Alveolar Macrophages

Alveolar macrophages from the first lung washings were discarded because they were presumed to be compromised metabolically. Electron microscopic observation of alveolar macrophages which were obtained from the first lavage indicated that the cells were, indeed, unhealthy. Generally, they lacked intact mitochondria, had disorganized endoplasmic reticulum, lacked good nuclear structure, and lysosomal particles (Figure 7). On the other hand, alveolar macrophages obtained from the second and third lavages had the appearance of macrophages that were alive and healthy (Figure 8). Ultrastructural examination of the non-encapsulated cryptococcal cells demonstrated that a small amount of capsule material remained on the cells (Figure 9). Also, it should be noted that the cryptococcal cells were probably alive because of the presence of a white ring between the cell wall and the capsular material as suggested by Edwards, Gordon, Lapa, and Ghoirse (1967).

Figure 7--Guinea pig alveolar macrophages from first lung lavage. 17,360x.

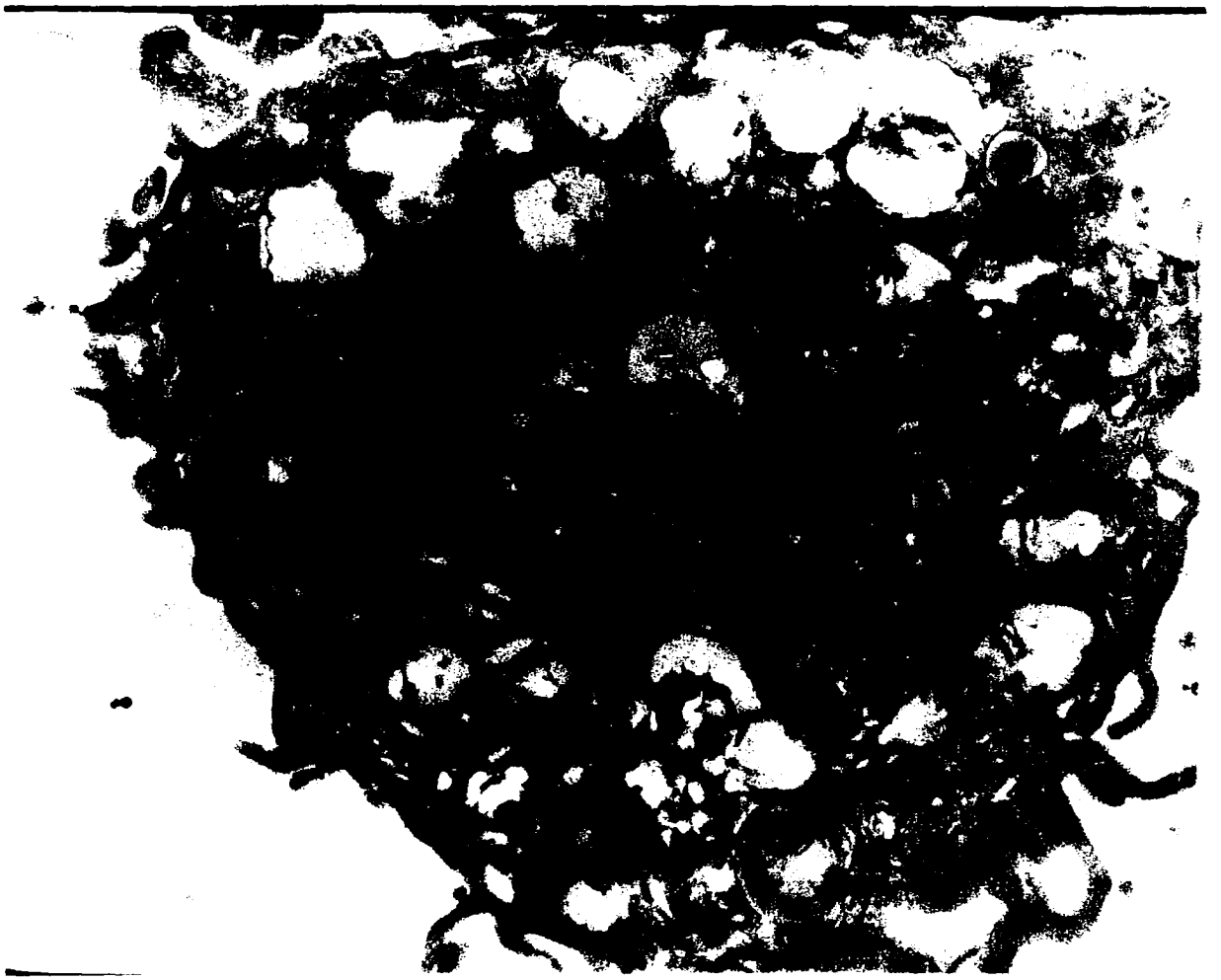

Figure 8--Guinea pig alveolar macrophages from second lung lavage. 8,400x. Nucleus (N), lysosome (L), and phagocytized material (P).

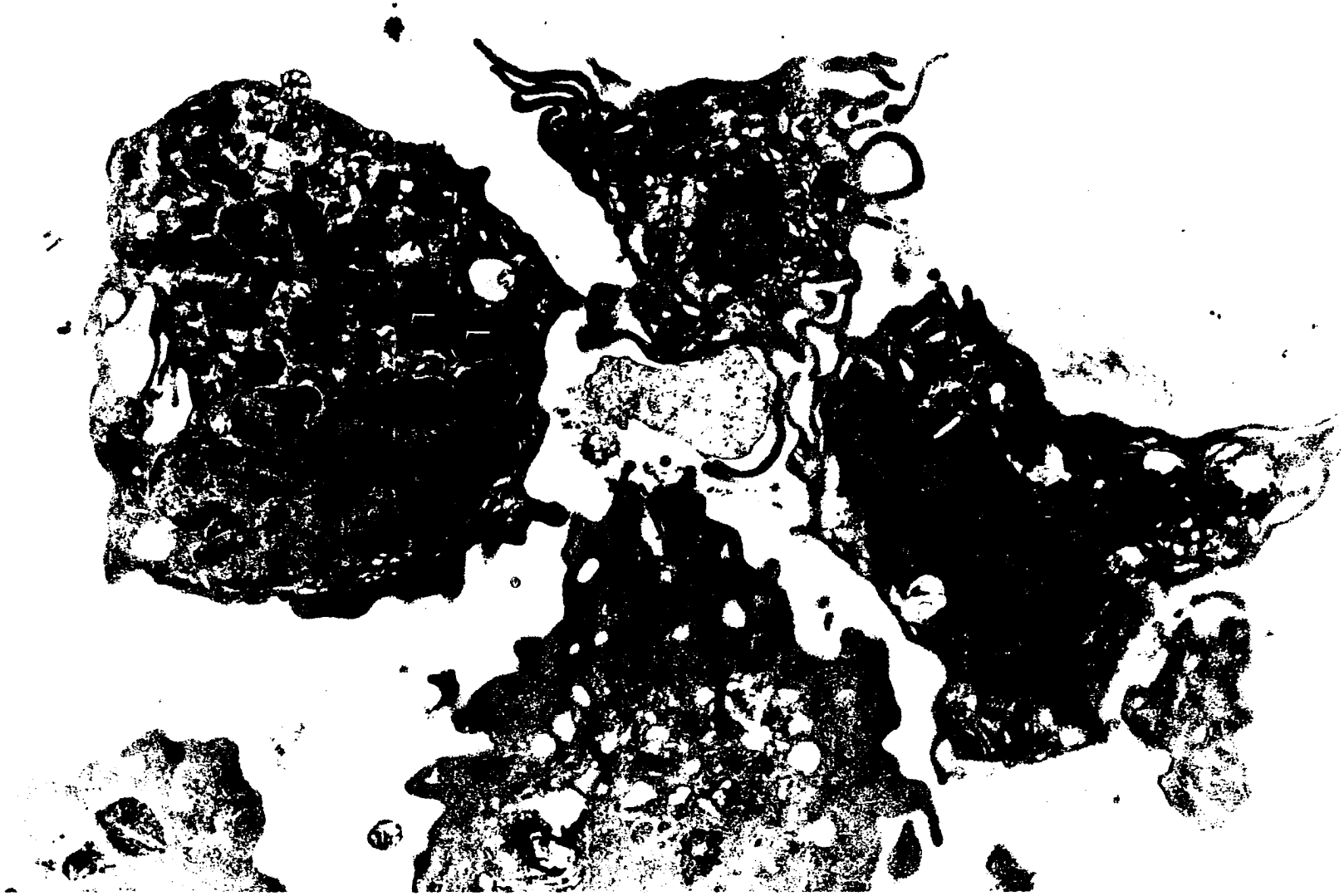

Figure 9--Budding non-encapsulated cryptococcal cell. 22,100x. Note white ring (R) around mother cell. These yeast cells were incubated with guinea pig alveolar macrophages for 2.0 hour *in vitro*. Note cell wall (CW), capsule (C), and lipid vacuole (V).


V
C
V
R
CW

Attempts were made to examine alveolar macrophages that had phagocytized cryptococcal cells *in vitro*. It was hoped that yeast cells would be seen inside phagosomes and that, perhaps, there would be evidence of lysosomes emptying their contents into the phagosome. Figure 10 shows a cryptococcal cell inside a guinea pig alveolar macrophages after 4 hours incubation in a typical phagocytosis experiment. The cell is budding and appears to be inside a vacuole. The EM grid blocked the view of other cellular components. However, even though the stain made the photograph foggy, no lysosomes appeared close to the organism. Moreover, because of the presence of the white line around the mother yeast cell, it appeared to be viable.

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. Before the *in vivo* fate experiments could be accomplished, it was necessary to devise a medium that allowed growth of cryptococcal cell with no capsule and in which the yeast cells were less than 5.0 μ in diameter (ordinarily growth in liquid media produces large cells and large capsules). Diameters of less than 5.0 μ would allow passage of cells into the alveolar space.

Table 3 shows the results of culturing *C. neoformans* in various modifications of basic Czapek medium. The basic

Figure 10--Budding cryptococcal cell phagocytized by a guinea pig alveolar macrophage. Note white ring (R), cell wall (CW), capsule (C), and phagocytic vacuole (P).

P
C
CW

TABLE 3

MODIFIED CZAPEK MEDIUM:
EXPERIMENTAL
MODIFICATIONS

Modifications	gm/100 ml	Growth Characteristics
1. $NaNO_3$	2.0	7-8μ diameter
Glucose	1.0	Capsule varies
K_2HPO_4	0.1	Growth good
2. $NaNO_3$	2.0	5-6μ diameter
Glucose	0.1	Small capsule
K_2HPO_4	0.1	Growth poor
3. $NaNO_3$	2.0	8-10μ diameter
Glucose	0.1	Large capsule
		Growth good
4. $NaNO_3$	3.0	9-10μ diameter
Glucose	0.1	Large capsule
		Growth good
5. $NaNO_3$	3.0	4.5-5.5μ diameter
Glucose	0.1	Small capsule
K_2HPO_4	0.1	Growth poor

Modified Czapek medium contains: $MgSO_4$, 0.5 gm/l; KCl, 0.5 gm/l; $FeSO_4$, 0.01 gm/l; Peptone, 5.0 gm/l; and yeast extract 1.0 gm/l.

Czapek medium, plus 3.0 gm/per cent $NaNO_3$, 0.1 gm/per cent D-glucose, and 0.1 gm/per cent K_2HPO_4, yielded small cells and small capsules, but poor growth. In other words, a small, lightly encapsulated cell could be produced, but its growth was poor.

Based on the observation that the K_2HPO_4 somehow decreased the size of the cells, a large series of modifications of Czapek medium were tested for their ability to produce small, preferably non-encapsulated cells, but with adequate growth. Table 4 contains the results of experiments in which varying concentrations of K_2HPO_4 were employed with an increased glucose concentration (200 mg/100 ml) in the basic Czapek medium. The medium containing 25 mg K_2HPO_4/100 ml, produced the most desirable cellular characteristics after 3 days of growth, i. e., adequate growth and no capsules, but the cells were too large. Table 5 shows the results obtained with the next set of modifications in which basic Czapek medium was prepared de novo, either without yeast extract, with yeast extract (100 mg/ml) and peptone (10 mg/100 ml), or with yeast extract (100 mg/ml). The K_2HPO_4 concentration of 50 mg/100 ml in the presence of peptone and yeast extract gave the best results, with only 10 per cent encapsulated cells, a cell diameter of 4.5 μ, and very good growth.

Even though the foregoing medium yielded good growth and cell size, an even smaller cell size was desirable.

TABLE 4

GROWTH CHARACTERISTICS AND DIAMETER OF *C. NEOFORMANS* GROWN IN DIFFERENT VARIATIONS OF CZAPEK MEDIUM*

K_2HPO_4 mg/100 ml	Growth Characteristics
0	4.25×10^6 cells/ml 6.0μ diameter 10% Encapsulated
25	6.3×10^6 cells/ml 5.0μ diameter Non-encapsulated
50	7.15×10^6 cells/ml 5.5μ diameter 70% Encapsulated
100	7.85×10^6 cells/ml 5.5μ diameter 70% Encapsulated
150	8.9×10^6 cells/ml 6.0μ diameter 100% Encapsulated

*containing glucose, 200 mg/100 ml.

TABLE 5

GROWTH CHARACTERISTICS AND DIAMETER OF *C. NEOFORMANS* GROWN IN DIFFERENT VARIATIONS OF CZAPEK MEDIUM

K_2HPO_4 mg/100 ml	Without yeast extract	With yeast extract	With peptone and yeast extract
0	No growth	8.1×10^6 cells/ml 6.0μ diameter Non-encapsulated	11.0×10^6 cells/ml 5.5μ diameter 10% Encapsulated
25	No growth	8.7×10^6 cells/ml 6.0μ diameter 20% Encapsulated	11.0×10^6 cells/ml 5.5μ diameter 10% Encapsulated
50	No growth	10.1×10^6 cells/ml 6.0μ diameter 20% Encapsulated	10.2×10^6 cells/ml 4.5μ diameter 10% Encapsulated
75	No growth	11.3×10^6 cells/ml 5.5μ diameter Non-encapsulated	10.5×10^6 cells/ml 6.0μ diameter 20% Encapsulated
100	No growth	13.1×10^6 cells/ml 5.25μ diameter Non-encapsulated	8.2×10^6 cells/ml 6.5μ diameter 20% Encapsulated
125	No growth	14.0×10^6 cells/ml 6.0μ diameter Non-encapsulated	11.4×10^6 cells/ml 5.0μ diameter 25% Encapsulated
150	No growth	14.0×10^6 cells/ml 6.0μ diameter Non-encapsulated	10.0×10^6 cells/ml 6.0μ diameter 30% Encapsulated

Table 6 shows the results obtained with another set of media variations. The media varied not only in K_2HPO_4 concentration, but also with respect to presence or absence of glucose. Examination of the cells revealed that the medium containing 50 mg/100 ml and Peptone without glucose yielded fair growth, no capsules, and desirable cell size (4.0 μ, range 3.6-4.7 μ).

Aerosolization of Radioactive Cryptococcal Cells

Several attempts were made to aerosolize the cryptococcal cells using the Tri-R infection apparatus and guinea pigs. Viable plate counts from the homogenized guinea pig lungs that received the radioactive cells showed only 150 organisms (av.) per lung at "0" time. No cryptococcal cells could be cultured from the lung 4.0 hours after aerosolization. Radioactivity decreased from 555 cpm at zero time to 435 cpm after 4 hours exposure. By dilution plate count of the nebulizer solution, the guinea pigs were exposed to approximately 2.52×10^7 organisms during the aerosolization time period. Obviously, only a very small percentage of the organisms reached the alveolar spaces. Although it appeared that the guinea pigs were able to clear the cryptococcal cells, the number of organisms inhaled was too small to draw any firm conclusions. Moreover, the radioactivity data were not significant since the background counts ranged from 360-420 cpm.

TABLE 6

GROWTH CHARACTERISTICS AND DIAMETER OF *C. NEOFORMANS* GROWN IN DIFFERENT VARIATIONS OF CZAPEK MEDIUM

K_2HPO_4 mg/100 ml	Peptone with glucose	Peptone without glucose
0	1.0×10^5 cells/ml 8.5μ diameter 100% Encapsulated	No growth
25	3.0×10^5 cells/ml 7.0μ diameter 100% Encapsulated	2.0×10^5 cells/ml 5.0μ diameter Non-encapsulated
50	7.0×10^5 cells/ml 6.5μ diameter 70% Encapsulated	9.0×10^5 cells/ml 4.0μ diameter Non-encapsulated
75	8.0×10^5 cells/ml 5.5μ diameter 40% Encapsulated	1.0×10^5 cells/ml 4.5μ diameter Non-encapsulated
100	1.4×10^6 cells/ml 6.0μ diameter 50% Encapsulated	1.2×10^6 cells/ml 5.0μ diameter Non-encapsulated
125	1.4×10^6 cells/ml 5.0μ diameter 40% Encapsulated	1.0×10^6 cells/ml 4.7μ diameter Non-encapsulated
150	1.6×10^6 cells/ml 5.5μ diameter 50% Encapsulated	1.0×10^6 cells/ml 4.5μ diameter Non-encapsulated

Transtracheal Instillation Experiments

Because of the problems encountered in the aerosolization experiments, it was decided to look for an alternate method to determine *in vivo* fate of *C. neoformans*. Consequently, it was decided that more cells could be deposited in the lungs if the organisms were injected directly into the distal part of the trachea.

This was accomplished by injecting approximately 1.5-1.7 x 10^7 viable cryptococcal cells into the lower trachea of the guinea pigs along with 2.0 cc of air. The animals were sacrificed either immediately or at varying time intervals post instillation by clamping the trachea. As in previous experiments, the lungs were removed, homogenized, scored for radioactivity, and the number of viable *C. neoformans* was determined. Table 7 shows the results of this study. It will be seen that the average number of viable cryptococcal cells dropped from 1.51 x 10^7 to 1.24 x 10^7 per whole lung over the 360 minute time period. The 90 minute and 360 minute data are also presented in Figures 11 and 12. A reduction in viability of only 18 per cent occurred after 6.0 hours incubation, with a concomitant decrease in ^{32}P counts of 14 per cent. Based on these observations, it appeared that the cryptococcal cells were not killed in the guinea pig lungs.

Fungicidal Activity of Myeloperoxidase

It has been shown that myeloperoxidase (MPO) extracted from human PMN's, in conjugation with a halide and hydrogen

TABLE 7

DEPOSITION AND CLEARANCE OF ^{32}P LABELED LOW-ENCAPSULATED C. NEOFORMANS FROM GUINEA PIG LUNGS

Time after Instillation	Animal Number	Number C. Neoformans/lung Cell count x 10^7	^{32}P- counts/lung CPM x 10^6
0 min	1	1.61	1.43
0 min	2	1.43 (1.51 av)	1.35 (1.37 av)
0 min	3	1.51	1.39
30 min	4	1.52	1.38
30 min	5	1.58 (1.52 av)	1.28 (1.36 av)
30 min	6	1.46	1.41
90 min	7	1.53	1.30
90 min	8	1.47 (1.50 av)	1.30 (1.27 av)
90 min	9	1.49	1.24
180 min	10	1.30	1.31
180 min	11	1.41 (1.38 av)	1.24 (1.27 av)
180 min	12	1.45	1.26
360 min	14	1.31	1.23
360 min	15	1.21 (1.24 av)	1.16 (1.19 av)

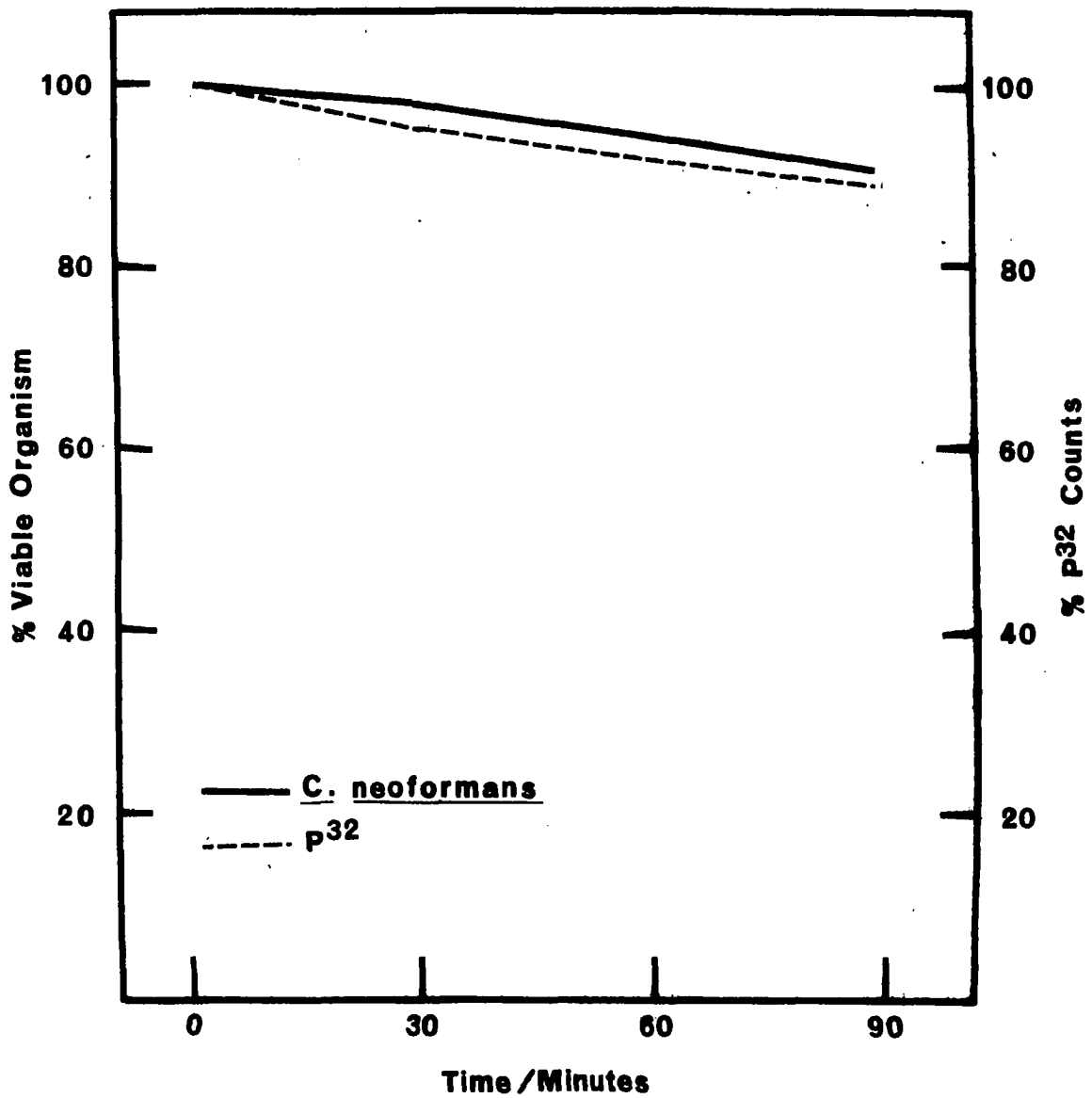

Figure 11--Deposition and clearance of ^{32}P labeled C. neoformans after transtracheal instillation.

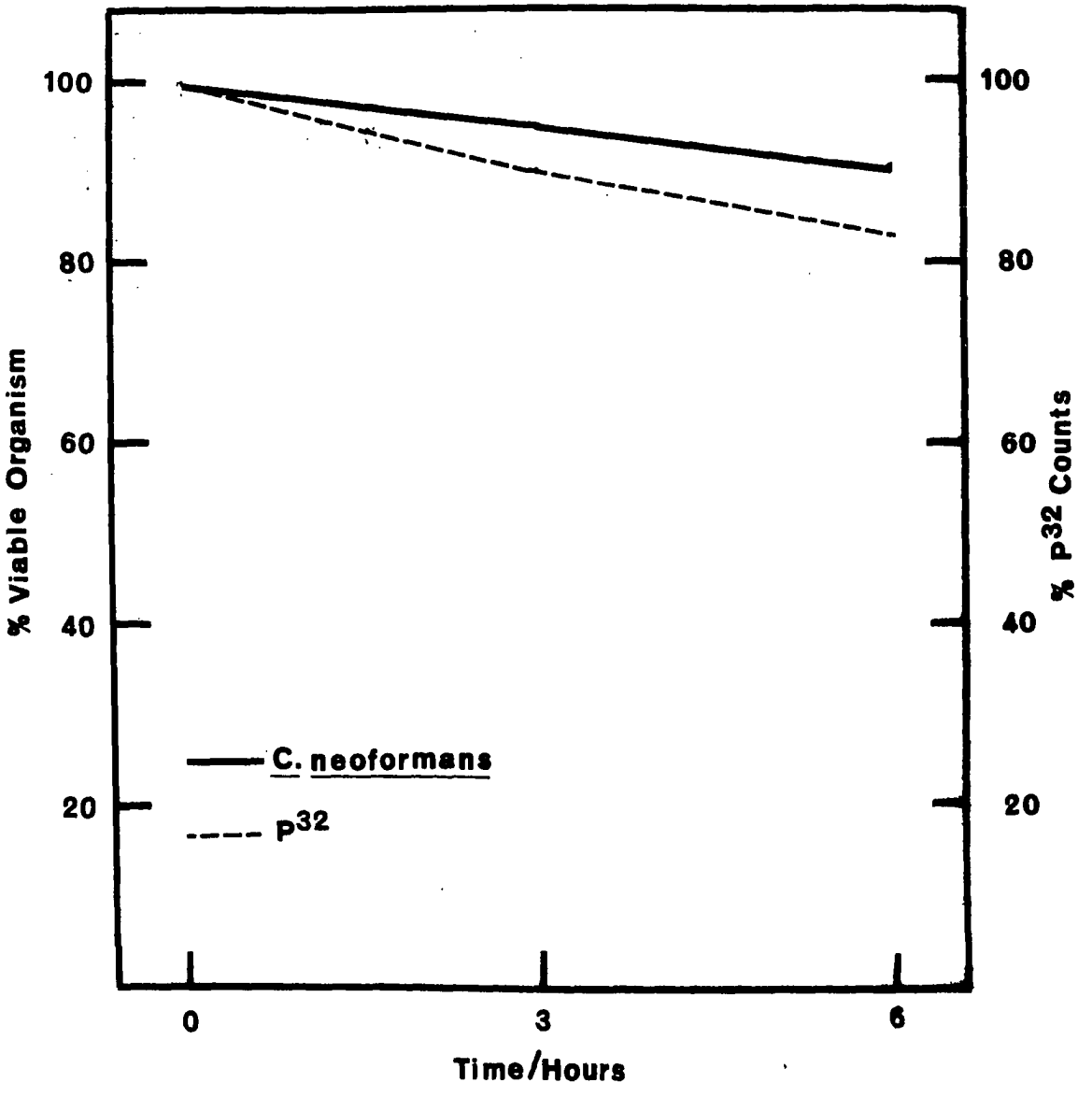

Figure 12--Deposition and clearance of ^{32}P labeled C. neoformans after transtracheal instillation.

peroxide, exhibits a bactericidal effect (Klebanoff, 1965; Lehrer, 1969). Except for C. albicans it is not known whether this MPO system can effectively kill various fungi, including C. neoformans.

To answer this question, a series of experiments was designed to test the ability of crude lysosomal extracts (containing MPO), a halide, and H_2O_2 to kill cryptococcal cells. Table 8 demonstrates dilution plate counts (obtained from the 1×10^{-3} dilution) when various organisms were tested against several combinations of the components of the MPO system. In these preliminary experiments, 470 units of MPO, 5×10^{-4} M H_2O_2, and 1×10^{-5} M NaI (final conc.) were used. Examination of Table 8 reveals that the combination of MPO-NaI-H_2O_2 killed encapsulated and non-encapsulated C. neoformans, C. albicans, and S. schenckii. It should also be noted that R. oryzae was not killed. Also, NaI-H_2O_2, NaI, or H_2O_2 by themselves were not effective in killing. However, a decrease was observed when the NaI-H_2O_2 combination was used against the cryptococcal cells.

Although these results were interesting, the studies lacked a good control for growth and the proper concentration of the halide and H_2O_2. Therefore, better controlled experiments were performed to answer conclusively whether or not the MPO-system could kill cryptococcal cells.

Table 9 demonstrates various conditions which were used to test the killing of encapsulated cryptococcal cells.

TABLE 8

EFFECT OF MYELOPEROXIDASE (MPO) SYSTEMS ON VIABLE COUNTS OF VARIOUS FUNGI

Organism	System	Viable Counts/min[2]				
		0	15	30	60	90
ClA[1]	MPO + H_2O_2	13.0	22.5	30.0	19.5	17.0
	NaI + H_2O_2	15.5	12.5	16.0	9.5	5.0
	MPO + NaI + H_2O_2	29.0	0.0	0.0	0.0	0.0
	H_2O_2	22.5	22.5	26.5	20.1	24.5
	NaI	21.5	21.0	15.5	19.0	20.5
Low pH[3]	MPO + H_2O_2	33.0	28.0	28.0	29.0	16.0
	NaI + H_2O_2	28.0	26.0	33.0	16.0	3.0
	MPO + NaI + H_2O_2	13.0	0.0	0.0	0.0	0.0
	H_2O_2	33.5	29.0	35.0	30.0	29.0
	NaI	24.0	18.0	24.0	26.0	26.0
C. albicans	MPO + NaI + H_2O_2	0.0	0.0	0.0	0.0	0.0
S. schenckii	MPO + NaI + H_2O_2	7.0	0.0	0.0	0.0	0.0
R. oryzae	MPO + NaI + H_2O_2	17.5	21.0	28.0	27.0	26.0

[1]Encapsulated *C. neoformans*
[2]Average count of two plates from 1 x 10^{-3} dilution
[3]Non-encapsulated *C. neoformans*

TABLE 9

EFFECT OF MYELOPEROXIDASE (MPO) AND SUPPLEMENTS ON THE VIABILITY OF ENCAPSULATED C. NEOFORMANS (CIA)

System	Halide M	MPO Units	H_2O_2 M	Percentage viable cells/min.			
				0	15	30	60
PO_4-Buffer pH 5.0	---	---	---	100.0	99.3	89.2	84.8
MPO	---	150	---	87.0	99.3	85.6	87.8
H_2O_2	---	---	$5.0x10^{-5}$	N. D.*	94.5	N. D.*	91.7
NaI	$1x10^{-4}$	---	---	83.5	88.5	84.1	76.2
H_2O_2 + NaI	$5x10^{-5}$	---	$5.0x10^{-5}$	82.2	75.3	N. D.*	83.5
MPO + NaI	$8.3x10^{-4}$	150	---	104.0	111.0	76.6	93.3
MPO + NaI + H_2O_2	$1x10^{-4}$	150	$5.0x10^{-5}$	0	0	0	0
NaCl + H_2O_2	$2x10^{-3}$	---	$3x10^{-3}$	84.1	78.1	84.8	0
NaCl + MPO	$2x10^{-3}$	150	---	105.3	89.9	116.6	106.8
NaCl	$2x10^{-3}$	---	---	82.1	100.0	84.5	86.9
MPO + NaCl + H_2O_2	$2x10^{-3}$	150	$3x10^{-3}$	100.7	100.6	78.7	33.3

*Not done

Tested separately, MPO, H_2O_2 and NaI, in the quantities used were not effective in killing encapsulated cells of C. neoformans (CIA). There was essentially no change in viability over a 60 minute time period when compared with the PO_4-buffer control. The combinations of NaI-H_2O_2 and MPO-NaI were also ineffective, since the percentage viability remained at 83.5 and 93.3, respectively, after 60 minutes. However, when the combination of MPO-NaI-H_2O_2 was incubated with the cryptococcal cells, there was immediate killing. It should be noted that death was recorded at zero time. Actually, zero time was approximately 5.0 minutes after all the components were mixed together. It required 5.0 minutes to dilution plate the reaction mixture and apparently this was enough time to allow for 100 per cent killing.

An attempt was made to substitute NaCl for the NaI. The combination of NaCl-H_2O_2 showed complete killing in 60 minutes (Table 9). The combination of MPO-NaCl-H_2O_2 showed approximately 67 per cent killing after 60 minutes. This seemed to indicate that Cl^- could be substituted for I^-. However, the final concentration of H_2O_2 was higher than in previous experiments (Table 8) and this may have accounted for the killing observed when NaCl and H_2O_2 were used alone. This observation also negates the possibility that MPO-NaCl-H_2O_2 is capable of killing C. neoformans because it must be assumed the H_2O_2 (3×10^{-3} M) was actually responsible for the death of the cells.

Table 10 demonstrates the results of a series of experiments similar to those seen in Table 9. The major difference was in the use of non-encapsulated cells. As before, MPO, H_2O_2, and NaI when used separately did not kill the organisms when compared to the buffer system. The combinations of NaI-H_2O_2, MPO-H_2O_2, and MPO-NaI, resulted in 81.3 per cent, 97.0 per cent and 81.8 per cent viability, respectively, after 60 minutes. The combination of MPO-NaI-H_2O_2 killed all the cells at zero time.

Because the concentration of H_2O_2 used in the experiments involving the NaCl (Table 9) was too high, the concentration was lowered when it was used with the non-encapsulated organisms. The results showed that no killing occurred after 60 minutes when MPO-NaI-H_2O_2 was incubated with the cryptococcal cells.

Klebanoff (1968) showed that myeloperoxidase could be separated from other bactericidal agents extracted from PMN's. This conclusion was based on the heat lability (100° C/10 minutes) and the inactivation by cyanide and azide. To test the possibility that MPO was not the agent which contributed to the death of cryptococcal cells, another series of experiments was devised. The results are shown in Table 11. The major point to be noted is that when MPO-NaI-H_2O_2 was incubated with KCN (3×10^{-3}M) or 3-amino triazole (3AT) (1×10^{-2}M) and encapsulated cells, no killing was observed. When MPO-NaI-H_2O_2 was incubated without either inhibitor,

TABLE 10

EFFECT OF MYELOPEROXIDASE (MPO) AND SUPPLEMENTS ON THE VIABILITY OF NON-ENCAPSULATED C. NEOFORMANS (CIA)

System	Halide M	MPO Units	H_2O_2 M	Percentage viable cells/min.			
				0	15	30	60
PO_4-Buffer pH 5.0	---	---	---	100.0	107.7	65.3	105.3
MPO	---	150	---	104.5	114.7	100.0	90.9
H_2O_2	---	---	$0.5x10^{-4}$	88.0	85.3	108.0	90.6
NaI	$1x10^{-4}$	---	---	78.0	104.0	93.3	106.6
H_2O_2 + NaI	$1x10^{-4}$	---	$0.5x10^{-4}$	100.0	100.0	120.0	81.3
MPO + H_2O_2	---	150	$0.5x10^{-4}$	98.0	108.7	93.2	97.0
MPO + NaI	$1x10^{-4}$	150	---	119.3	N. D.*	84.0	81.8
MPO + NaI + H_2O_2	$1x10^{-4}$	150	$0.5x10^{-4}$	0	0	0	0
MPO + NaCl + H_2O_2	$1x10^{-3}$	150	$0.5x10^{-4}$	123.6	101.3	75.0	91.6

*Not done

TABLE 11

EFFECT OF VARIOUS INHIBITORS ON THE MYELOPEROXIDASE (MPO) SYSTEM USING ENCAPSULATED C. NEOFORMANS (CIA)

System	KCN M	3AT[1] M	Heated MPO Units	Non-heated MPO Units	Percentage viable cells/min.	
					0	30
PO_4-Buffer pH 5.0	---	---	---	---	100.0	100.0
MPO	---	---	---	44	100.0	157.0
MPO	---	---	44	---	100.0	101.0
MPO + NaI + H_2O_2	---	---	---	44	0.0	0.0
MPO + NaI + H_2O_2	---	---	44	---	100.0	114.0
NaI + H_2O_2 + KCN	$3x10^{-3}$	---	---	---	100.0	77.9
MPO + NaI + H_2O_2 + KCN	$3x10^{-3}$	---	---	44	100.0	92.1
MPO + NaI + H_2O_2 + 3AT	---	$1x10^{-2}$	---	44	100.0	105.1
NaI + H_2O_2 + 3AT	---	$1x10^{-2}$	---	---	100.0	83.1

[1]3 amino-triazole

killing occurred immediately. If the MPO was heated to 100° C for 10 minutes, this also effectively inhibited killing by the MPO-system. Also, in this set of experiments, the concentration of MPO was decreased to 44 units without effecting its activity. The combination of NaI-H_2O_2-3AT and NaI-H_2O_2-KCN had no effect on the viability of the cryptococcal cells.

Table 12 demonstrates results similar to those shown in Table 11. The major difference was that non-encapsulated cryptococcal cells were used instead of encapsulated cells. Again, KCN, 3AT, and heated MPO inhibited the killing power of the MPO-halide-H_2O_2 system. As before, only 44 units of MPO were employed in the MPO-system.

Assay of Myeloperoxidase from Various Leucocyte Extracts

Since the MPO-system efficiently killed cryptococcal cells, it was decided to look for the enzyme in alveolar macrophages. Guinea pig and human alveolar macrophages were disrupted and treated in a manner similar to that used with the human peripheral leucocytes from leucopacs. After disruption, extracts were assayed for the presence of MPO. Figure 13 shows the results of these assays. It is noted that extracts from human peripheral leucocytes extracts number 6 and 7 (0.1 ml) caused an increase in optical density when incubated with H_2O_2 and 1 per cent o-dianisidine. Using the same substrate, purified horse-radish peroxidase (Worthington, Biochemical, Freehold, New Jersey) also caused

TABLE 12

EFFECT OF VARIOUS INHIBITORS ON THE MYELOPEROXIDASE (MPO) SYSTEM USING NON-ENCAPSULATED C. NEOFORMANS (CIA)

System	KCN M	3AT[1] M	Heated MPO Units	Non-heated MPO Units	Percentage viable cells/min.	
					0	30
PO_4-Buffer pH 5.0	---	---	---	---	100.0	100.0
MPO	---	---	44	---	100.0	97.3
MPO	---	---	---	44	100.0	96.2
MPO + NaI + H_2O_2	---	---	---	44	00.0	00.0
MPO + NaI + H_2O_2	---	---	44	---		
NaI + H_2O_2 + KCN	$3x10^{-3}$	---	---	---	100.0	120.5
MPO + NaI + H_2O_2	---	---	---	44	00.0	00.0
MPO - NaI - H_2O_2 - KCN	$3x10^{-3}$	---	---	44	100.0	87.9
NaI - H_2O_2 - 3AT	---	$1x10^{-2}$	---	---	100.0	96.7
MPO - NaI - H_2O_2 - 3AT	---	$1x10^{-2}$	---	44	100.0	97.5

[1] 3 amino-triazole

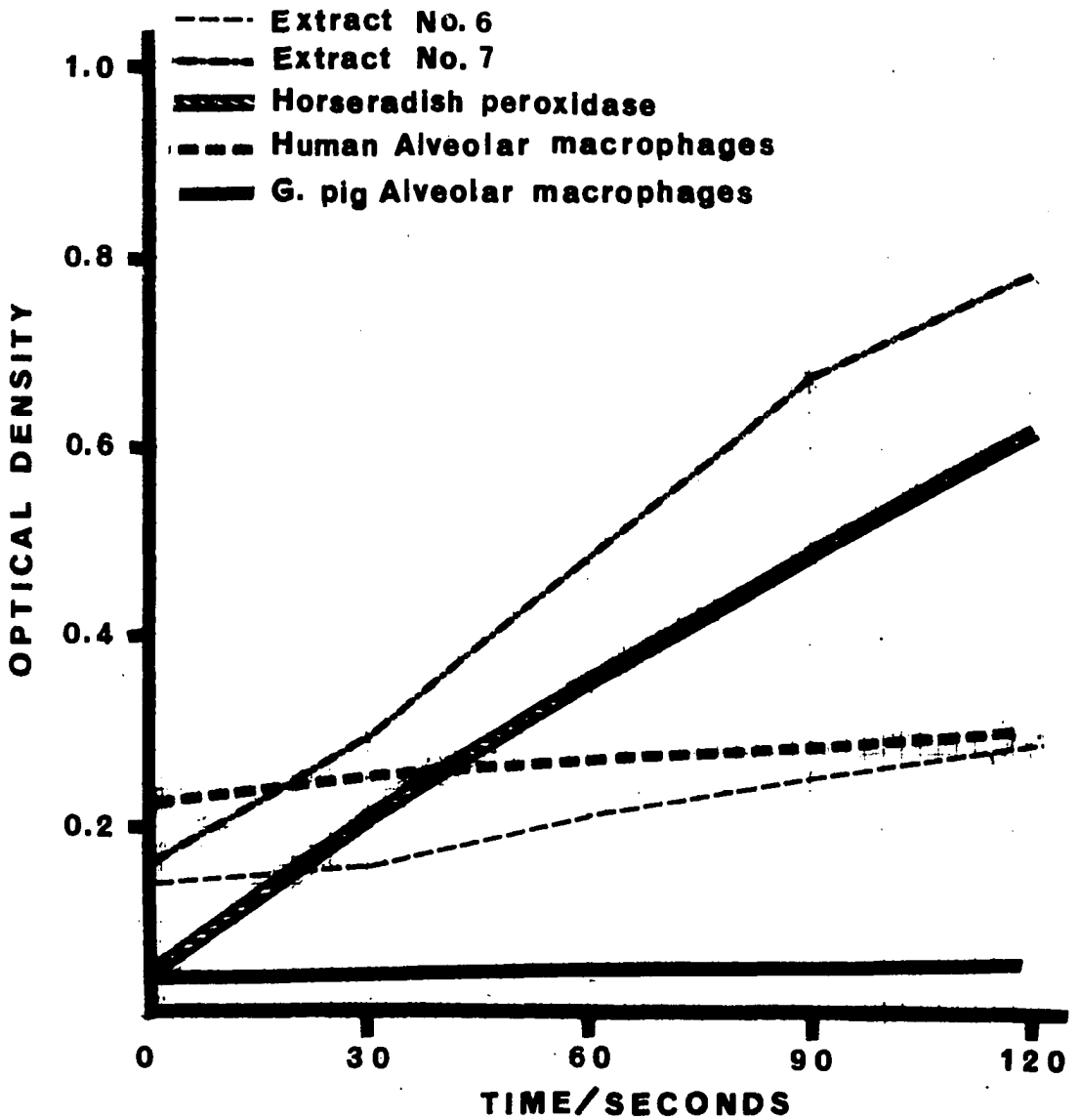

Figure 13--Assay for myeloperoxidase extracted from various sources. Increase in absorbance versus time in seconds.

an increase in O. D. When extracts from guinea pig and human alveolar macrophages were tested with the same substrates very little increase in optical density was observed with guinea pig alveolar macrophages. The human alveolar macrophages preparation showed an increase, but the preparation contained 11 per cent PMN's and an undetermined number of red blood cells. Converting increase in O. D. versus time to o-dianisidine units, extracts 6 and 7 contained 748 and 4900 units/ml, respectively. Guinea pig alveolar macrophages contained 110 units/ml and human alveolar macrophages contained 132 units/ml. The purified horse-radish peroxidase contained 2020 units/ml.

CHAPTER IV

DISCUSSION

It was not surprising to find that guinea pig alveolar macrophages can phagocytize non-encapsulated cryptococcal cells. It has already been shown that human PMN's are capable of phagocytizing cryptococci (Bulmer and Sans, 1967). Since alveolar macrophages are the phagocytes of the lung, one would expect to observe good phagocytosis of particles entering the lung. However, it appears that phagocytosis by alveolar macrophages *in vitro* is somewhat lower than that seen with PMN's (Bulmer and Sans, 1967). Maximal phagocytosis ranged between 57-60 per cent for alveolar macrophages, while human PMN's averaged about 72 per cent. However, comparison of the two cell lines may not be valid for obvious reasons. It was interesting to note that optimal phagocytosis was influenced by serum concentration and yeast cell to alveolar macrophage ratio (Figures 1 and 2). Human PMN's can phagocytize with surprising efficiency in the apparent absence of serum (Tacker, *et al.*, 1972).

Capsular polysaccharide from *C. neoformans* impairs phagocytosis by guinea pig alveolar macrophages (Table 1).

It appears that as little as 100 µg/ml can effectively reduce phagocytosis *in vitro* to almost one-half of the normal value. Higher levels of polysaccharide (up to 1800 µg/ml) were only slightly more effective than the 100 µg level. This was not an unpredictable finding since Bulmer and Sans (1968) showed the same thing using human PMN's. It is important, however, because it had been reported previously that non-encapsulated *C. neoformans* can produce significant amounts of capsular material in human lung tissue after 4.0 hours incubation (Farhi, 1969).

Intracellular fate studies demonstrated that guinea pig alveolar macrophages cannot kill phagocytized cryptococcal cells within 4.0 hours *in vitro* (Figure 3). There was a decrease in viability, both with and without alveolar macrophages, but this was apparently due to an anticryptococcal serum factor (Tacker, *et al.*, 1972). This is in contrast to the finding that human PMN's are effective in killing *C. neoformans* (Tacker, *et al.*, 1972; Diamond, Root, Bennett, 1972). Human blood monocytes have been shown to be relatively ineffective in killing *C. neoformans* (Diamond, *et al.*, 1972), however blood monocytes are metabolically different from alveolar macrophages (Karnovsky, 1962; Oren, Farham, Saito, Milofsky and Karnovsky, 1963). It is possible that the alveolar macrophages were metabolically compromised owing to the experimental procedures used, thus leaving them incapable of killing ingested organisms. This possibility was

ruled out, however, by showing that they could effectively kill E. coli within 2.0 hours (Figure 4) and that 84 per cent of them appeared to be viable by the trypan blue exclusion test. This finding is in contrast to that reported by Pavillard (1963), who reported that rat alveolar macrophages were ineffective in killing intracellular E. coli strain BV. However, Pavillard used tissue culture alveolar macrophages and a different strain of E. coli than the one employed in the present experiments. Furthermore, it has been shown by Cohn and Morse (1959), in the case of staphylococci, that strain variation influences the ability of the leucocyte to kill ingested organisms.

It has been shown by Myrvik, et al. (1961a), Evans and Myrvik (1967) and Hirsch and Cohn (1960) that rabbit alveolar macrophages can be induced to a more active state. Such activated cells contain larger quantities of various catabolic enzymes, mitochondria, lysosomes, and other cells organelles. Activation of alveolar macrophages may be achieved by parenteral injection of endotoxin, gram negative bacteria, and BCG in oil. It is possible that cryptococcal cells are not killed by alveolar macrophages because the macrophages lack high levels of the necessary enzymes, i.e., they are not sufficiently activated.

An attempt was made to activate guinea pig alveolar macrophages by injecting animals with heated killed S. typhosa and S. marcescens. The results showed that a degree

of activation occurred as evidenced by an increase in enzyme levels (Table 2). However, when these activated alveolar macrophages were tested for their ability to kill C. neoformans, they were found to be ineffective (Figures 5 and 6).

Phagocytosis of cryptococcal cells by human alveolar macrophages could not be demonstrated. It was hoped that collecting alveolar macrophages from a recently expired patient would yield healthy cells. The lungs used in these experiments were lavaged for alveolar macrophages 5.0 hours postmortem. Only 40 per cent of the cells were viable and the percentage phagocytosis was very low. It seems quite certain that the low percentage of phagocytosis can be attributed to lack of metabolic integrity. Cohen and Cline (1971) were able to culture human alveolar macrophages from fresh, surgically resected lungs. These cells were able to phagocytize heat killed C. albicans and viable Listeria monocytogenes. They were also able to kill the L. monocytogenes, but not C. albicans blastospores.

It was interesting to note that the electron micrographs showed that the alveolar macrophages from the first lavage appeared to be degenerated (Figure 7). These cells probably represent the so-called "dust cells" which are dying cells residing in the upper respiratory tract. From the second and third lavage of the lungs, cells were collected which had more typical morphology and which appeared to have a good complement of lysosomes (Figure 8). These were the

cells used in all *in vitro* phagocytosis and fate experiments.

One of the objectives of these experiments was to observe the ultrastructure of cryptococcal cells after they had been incubated with guinea pig alveolar macrophages. Examination of an engulfed, budding cryptococcal cell showed that the cell was probably alive and was situated in a phagocytic vesicle (Figure 10). Lysosomes could not be found near the phagocytic vesicle. It is difficult to know how long the cell had been in the phagocyte, but it is tempting to conclude that, in response to *C. neoformans*, the lysosomes do not empty their enzymes into the phagocytic vesicle.

In preparation for *in vivo* fate experiments, it was necessary to devise a medium which would support growth of large quantities of small (less than 5.0 μ) non-encapsulated cells of *C. neoformans*. Farhi (1969) showed that a high concentration of a salt ($NaNO_3$) in liquid medium would produce a cryptococcal cell with little or no capsule. A medium was therefore devised based on results obtained with different concentrations of $NaNO_3$, glucose, peptone, yeast extract, and K_2HPO_4. This medium produced the desired results, although it is difficult to speculate on why a basic medium containing peptone, 50 mg K_2HPO_4/100 ml, and no glucose yielded the best result (Table 5). It is possible that the glucose could be used directly in synthesis of capsule, however the combination of peptone and K_2HPO_4 was merely empirical.

Using cryptococcal cells grown in the special medium, an attempt was made to aerosolize the cells so that they could be inhaled into the lungs of guinea pigs. Even though large numbers of small cells (5.0 μ) were nebulized in the Tri-R Infection Apparatus, only a very few were retained by the guinea pig lungs. It is possible that the yeast cells were too large to enter the respiratory tract of the animals, thus accounting for the lower viable counts than were expected. Alternately, it could be concluded, however, that the cells were inhaled and immediately killed. If this hypothesis were true, one would expect to see a high radioactivity count in the lung tissue, since the cells were labeled with ^{32}P. This was not the case, since very little radioactivity was detected in the homogenized lungs. It is possible that, when the cryptococcal cells were nebulized, they picked up a film of water, or adhered together, which would have increased their size to greater than 5.0 μ. It has been shown by Hatch (1961) that particles must be less than 5.0 μ in diameter to reach the alveoli. Moreover, since the aerodynamic qualities of a particle include not only diameter, but also the density and shape, hence the measurement of a particle under the microscope provides insufficient information to predict its airborne behavior.

Since aerosolized cryptococcal cells could not be detected in adequate numbers in the lungs of guinea pigs, an alternate method was instituted. This method involved

instillation of fairly large numbers of non-encapsulated cells directly into the lungs of guinea pigs by way of the trachea. A small amount of air was also injected with the cells to help force the material directly into the bronchi and on to the alveoli. Results showed that large numbers of ^{32}P labeled cryptococcal cells were deposited in the lungs. In these experiments, the cryptococcal cells were not killed after 6.0 hours. Since the radioactivity remained high in the lung tissue, the cells apparently remained in the lungs (Figures 11 and 12). From this, it can be concluded that C. neoformans is not cleared from guinea pig lungs and is not killed after coming in contact with the lung milieu in the time period tested. Although the method of deposition used in these experiments was not as natural as voluntary inhalation, it did overcome the aerosol dynamics problem.

Myeloperoxidase (MPO), in combination with a halide and H_2O_2, has been shown to be an effective bactericidal and fungicidal element (Klebanoff, 1968; Lehrer, 1969; Lehrer and Cline, 1969). It was decided, therefore, to determine whether C. neoformans can be killed by the myeloperoxidase system. The first experiments showed that the combination of myeloperoxidase-NaI-H_2O_2 could kill C. neoformans in vitro (Table 8). Because MPO systems were known to kill C. albicans, other Candida spp., Saccharomyces cerevisiae, Rhodotorula spp., Geotrichum candidum, Aspergillus fumigatus,

and A. niger, it was decided to test the system with C. albicans. S. schenckii and R. oryzae were also tested in the MPO system. As was expected, the MPO system killed C. albicans and S. schenckii. MPO did not kill R. oryzae, however, thus indicating that not all fungi are killed by the MPO system (Table 8).

It required several trials to find the optimal concentrations of components that could be used for testing the MPO system in vitro. If too high a concentration of either NaI or H_2O_2 were used cryptococcal cells were killed in the absence of myeloperoxidase. Klebanoff (1965) was able to substitute NaCl for NaI as the halide in the MPO system and retain killing of E. coli. In the concentrations used in the present studies, it appeared that the same was true for C. neoformans; however examination of the data (Table 8) revealed that the concentration of H_2O_2 was too high and was causing cell death by itself. When the concentration of H_2O_2 was adjusted (Table 10) it was found that NaCl could not be substituted for NaI in the MPO system.

Overall, it can be concluded that (1) MPO-H_2O_2-NaI kills encapsulated and non-encapsulated C. neoformans equally well (Tables 9 and 10), (2) combinations of the other components alone or mixed did not kill C. neoformans appreciably, (3) KCN and 3AT, at the molar concentrations used, inhibited the MPO-NaI-H_2O_2 system (Tables 11 and 12), and (4) lower (44 units) levels of MPO were as effective in killing

as higher (150 and 470 units) levels (Tables 8, 11 and 12).

Although it is agreed by most researchers that myeloperoxidase systems are probably antimicrobial, the mechanism of this reaction is not clear. Some investigators feel that iodination of the microorganism is responsible, but this has not been shown conclusively (Klebanoff and Hamon, 1972). Others feel that chloride is the major halide ion involved which results in the formation of an aldehyde (Zglicynski, Stelmaszynska, Ostrowski, Naskalski, and Sznajd, 1968; Sbarra, Paul, Jacobs, Strauss and Mitchell, 1972a). Most workers do not agree on the levels of halide and H_2O_2 present in the phagocyte, nor do they agree that these levels would be attainable in the normal in vivo situation (Klebanoff and Hamon, 1972; Sbarra, et al., 1972b).

If a myeloperoxidase system is operative in alveolar macrophages, it would seem that cryptococcal cells would be killed after phagocytosis. Examination of the data (Figure 13) revealed that extracts from guinea pig alveolar macrophages lacked myeloperoxidase, while extracts from human peripheral leucocytes yielded high quantities of MPO. Human alveolar macrophages seemed to have MPO activity, but the extracts were contaminated with PMN's and RBC's, known to be rich in peroxidases. Cohen and Cline (1971) have shown, by chemical stain only, that human alveolar macrophages lack myeloperoxidase.

If alveolar macrophages truely lack MPO, it would be tempting to conclude that C. neoformans is not killed by these cells because they lack an MPO system. It should be emphasized, however, that MPO is not the only antimicrobial system, as pointed out earlier in the manuscript. Recently, Lehrer (1972) has described a candidicidal mechanism in neutrophils of subjects with hereditary myeloperoxidase deficiency. This system seems to operate independently of MPO, iodination, or the direct effect of H_2O_2.

CHAPTER V

SUMMARY

In vitro phagocytosis studies indicated that guinea pig alveolar macrophages could phagocytize, but not kill engulfed non-encapsulated cells of *C. neoformans*. Phagocytosis was shown to be inhibited by as little as 100 µg/ml of cryptococcal capsule material. Activated macrophages were unable to kill engulfed cryptococcal cells after 4.0 hours incubation *in vitro*. Fate studies conducted with intact animals indicated that their lungs were incapable of clearing ^{32}P-labeled non-encapsulated *C. neoformans* after 6.0 hours. The lungs also lacked the ability to kill the cryptococcal cells after transtracheal instillation.

Myeloperoxidase (MPO) extracted from human polymorphonuclear leucocytes was effective in killing *C. neoformans* when coupled with sodium iodide and hydrogen peroxide in the proper concentrations. Inhibition of the MPO system was accomplished by treatment with potassium cyanide, 3-amino triazole, or heat. Attempts to substitute sodium chloride for sodium iodide in the MPO system failed.

Myeloperoxidase activity was demonstrated in extracts from human polymorphonuclear leucocytes. MPO activity could not be detected in extracts from human and guinea pig alveolar macrophages.

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