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THE UNIVERSITY OF OKLAHOMA

GRADUATE COLLEGE

ROLE OF MACROPHAGES IN IMMUNITY AND PATHOGENESIS OF EXPERIMENTAL CRYPTOCOCCOSIS INDUCED BY THE AIRBORNE ROUTE

A DISSERTATION

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RIAD MOHAMAD KARAOUI

Norman, Oklahoma

1975

ROLE OF MACROPHAGES IN IMMUNITY AND

PATHOGENESIS OF EXPERIMENTAL CRYPTOCOCCOSIS

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ROLE OF MACROPHAGES IN IMMUNITY AND PATHOGENESIS OF EXPERIMENTAL CRYPTOCOCCOSIS INDUCED BY THE AIRBORNE ROUTE

CHAPTER I

INTRODUCTION

Cryptococcosis is a systemic mycotic infection caused by the yeast-like fungus, Cryptococcus neoformans. The organism has been found in soils contaminated with bird and animal excrement which support the growth of C. neoformans. The organism reproduces when climatic conditions are satisfactory, followed by release and dissemination of infectious aerosols which gain entrance into the respiratory tract through inhalation (29). C. neoformans produces a polysaccharide capsule and may cause subacute or chronic infection in humans and animals. Cryptococcosis frequently involves the lungs with occasional dissemination to the brain, meninges, kidneys, skin and bones. Dissemination occurs although resistance factors to growth of Cryptococcus have been described in human serum, saliva, and cerebrospinal fluid (26).

Pathogenesis of Cryptococcus neoformans has been elucidated by studies conducted in experimental animals through the use of various routes of infection. Kong and Levine (28) found that LD_{50} for

C. neoformans in inoculated mice increased in the following order: intracerebrally, intravenously, and intraperitoneally. Pappagianis (49), however, demonstrated that the previous three routes of infection allowed rapid dissemination of C. neoformans to the internal organs. When the lungs were the portal of entry, primary pulmonary disease was initiated and dissemination to internal organs was delayed. Intranasal instillation technique, also, has been used to induce cryptococcosis. The inoculum in this technique must be administered in a fluid suspension directly into the nostrils of anesthetized animals (54). Extreme care must be followed in intranasal instillation investigations to prevent animals from contracting fluid pneumonia not directly attributable to the yeast-like fungus; and to prevent contamination of the environment by aerosols generated by induced sneezing of inoculated animals. Ritter and Larsh introduced two drops of inoculum containing 10,000 viable yeast cells into one nostril of male white mice (54). After this intranasal instillation of the inoculum, 50% of mice died within a 12 week period. The first mortality, macroscopic lesions, and dissemination occurred during the fourth week after exposure. These results supported the theory that the respiratory tract was probably the natural portal of entry of C. neoformans into the host.

Smith et al. (59) have observed that mice infected by the aerosol route were positive to cryptococci in all organs cultured. The disease was progressive and analogous to that of the disease progression in human cryptococcosis. Larsh (29) proved that inbred mice (BALB c/j) were susceptible to Cryptococcus neoformans 184 by the airborne route of

inoculation. Experimental results obtained in this study indicated that C. neoformans was isolated most frequently from the lungs, followed by the liver, spleen, brain and heart. All tissues were negative from animals sacrificed at 24, 48, 72 hours and at 1 and 2 weeks, with the exception that in the latter periods, 67% of the lungs were infected with C. neoformans.

Studies by Mackaness (33), Elberg (18) and Mitsuhashi (41) have demonstrated that cellular factors play the decisive role in acquired resistance to facultative intracellular bacterial infections such as tuberculosis, brucellosis, listeriosis and salmonellosis. Appropriate stimulation of these host factors by a given intracellular bacterium also protected experimental animals against subsequent challenge with different strains of homologous organism (11), as well as against challenge with unrelated intracellular bacterial species (17). Gentry, Remington, and Ruskin (20, 53, 55, 56) proved that cross-protection among intracellular organisms extends beyond phylogenetic lines to include not only bacteria, but a number of diverse intracellular organism such as protozoa, viruses, and fungi.

The defense mechanism of man in systemic mycotic infections has not been well defined. Antibodies, arising during the course of the disease, have high diagnostic and prognostic values, but their protective role has not been demonstrated (28). Many studies, however, have indicated that cell mediated immune responses play a major role in resistance against the mycotic infections. Chilgren et al. (9) has described patients with chronic mucocutaneous candidiasis due to

impairment of lymphocyte function associated with cell mediated immunity. Miya and Marcus, 1961, (42) reported that prior vaccination of mice with H. capsulatum increased the fungicidal capacity of macrophages. In other studies, Howard et al., 1971, (25) have shown that thymus dependent lymphocytes mediate macrophage suppression of intracellular growth of the organism in vitro. Adamson and Cozad (1) observed that guinea pigs treated with anti-lymphocyte serum were more susceptible to development of fatal infection with H. capsulatum. Coccidioidal arthrospores and spherules were found in lung macrophages during the first two days after infection of nonvaccinated and vaccinated mice with Coccidioides immitis. Subsequently, the numbers of ingested and extracellular organisms declined more rapidly in the vaccinated than in the control animals, but actual intracellular killing of spherules by phagocytes was not ascertained (27). Spencer and Cozad reported that mice with delayed hypersensitivity to Blastomyces dermatitidis were protected from a lethal i.p. challenge with viable yeast cells of the organism (60). Mitchell and Friedman (40), Bulmer and Sans (5), have observed that Cryptococcus neoformans cells with small capsules were more easily phagocytized than those with large capsules. Adamson and Cozad (1) also proved that treatment of mice with anti-lymphocyte serum causes an increased fatality in mice infected with the organism, and in these animals more than 150 times as many parasites were present in the spleen. Diamond and Bennett, 1973, (14) found decreased lymphocyte transformation in patients with disseminated cryptococcosis.

Metchnikoff (39) suggested that the macrophage was the host's main defense against particulate foreign matter and many chronic infections. Other studies have demonstrated that the macrophage responds to certain infections with an adaptive increase in its defensive capacities (37, 48). Mackaness has shown that the enhancement of macrophage antimicrobial function during infection has an immunologic basis (34) which involves the sensitive lymphocyte (35). The mechanism of interaction between macrophage and lymphocyte during the expression of cellular immunity has not been well defined. However, the antigen activated thymus-dependent lymphocyte has been shown to be the immunologically specific cell which produces a number of soluble mediators called lymphokines (46, 47). These lymphokines include macrophage activating factor which may induce the activation of the macrophage which leads to the degradation of intracellular parasites due to increased synthesis of lysosomal enzymes (19, 46, 47). Mackaness described the characteristics of the "activated macrophages" obtained from animals during a certain stage of infection as increased spreading on glass surfaces, more heavily endowed with mitochondria and lysosomes, as well as displaying higher phagocytic and microbicidal activities than normal macrophages (36). The exact reasons for this difference between cells of normal and infected animals have not been clearly documented, nevertheless, evidence does support the assumption that the difference does not lie in specific antibacterial antibodies (4).

As cellular immunity decreases with time, it can be recalled in an accelerated manner only by repeated presentation of the original antigenic stimulus (34). The capacity to develop activated macrophages can

be transferred with sensitized lymphocytes (35), and anti-lymphocyte serum will prevent such transfer (38). When spleen lymphocytes from BCG-resistant mice were transferred into normal mice, the animals developed resistance to BCG but not to Listeria monocytogenes. Thus, the transferred resistance appears to be specific. However, if a small number of BCG organisms were present with the lymphocytes transferred to normal mice, the animals became resistant to subsequent challenge with L. monocytogenes. This suggests that the lymphocytes must be stimulated with the specific organism before non-specific manifestations can be observed (35).

The increased morbidity and mortality due to infection with C. neoformans among immunologically compromised patients were well documented (8, 45). Also, it was observed that amphotericin B and 5 - fluorocytosine fail to cure patients with cryptococcal infections despite the presence of sensitive organisms and relatively high levels of drug in blood and cerebral-spinal fluid (63). Therefore, dissemination of the organism and death of immunologically difficient patients occurred despite administration of therapeutic measures which prove adequate in similar patients. This ineffectiveness of drug therapy may be due to a failure of host defense mechanism against Cryptococcus neoformans.

It has been suggested that susceptibility to cryptococcal infections was associated with impaired immunologic defenses, particularly of the cell-mediated type (38). About 50% of patients with cryptococcosis have underlying illness, such as malignancy or disorders requiring chemotherapy which impairs cell-mediated immune functions (30, 61).

Yet, little is known about the lymphocyte-mediated immune responses to Cryptococcus neoformans. This paucity of knowledge has been engendered in part by the need of fungal antigens suitable for testing cell-mediated immune functions.

The strong association of cryptococcosis with underlying disorders of cell-mediated immunity has promoted efforts to identify cellular immune reactions to cryptococci (2). Initial work with protein containing extract of cryptococci produced positive skin test reactions in over one-half of patients with cryptococcosis. The diagnostic value of this extract was limited by extensive cross-reaction in patients with histoplasmosis and coccidioidomycosis, and failure to produce in vitro lymphocyte blastogenesis. The latter problem, however, was solved by the use of whole cryptococcal cell suspensions, but these preparations produced strong transformation cross-reactions in cryptococcin skin test negative subjects (14). Graybell (22) prepared cryptococcal antigen containing mostly polysaccharide and small amounts of proteins or polypeptides. This cryptococcin was potent and antigenically specific for cell-mediated immune assays. Strongly positive and specific lymphocyte transformation occurred in the presence of this cryptococcin in one-half of patients who had recovered from cryptococcosis. In contrast, few healthy subjects had positive transformation responses to cryptococcin. Bates (3) observed the development of delayed hypersensitivity to cryptococcin C - 184 as determined by footpad testing. Hypersensitivity reactions were first detected at 25 days following respiratory infection of BALB/cj mice with 3.2×10^3

viable C. neoformans cells (isolate 184). Footpad thickness steadily increased and the mean remained at a substantially high level through the termination of the experiment of 94 days.

An awareness in recent years of the prevalence of air-borne fungi and their high frequency as etiological agents of pulmonary diseases with potential dissemination to different organs of the body, has stimulated an interest in their pathogenesis and immunity. In vitro studies on resistance to C. neoformans have suggested the importance of macrophages interacting with lymphocytes in this infection (20). However, the role of alveolar and peritoneal macrophages in pathogenesis and immunity of Cryptococcus neoformans has never been studied during different stages of cryptococcal infection. Therefore, the purpose of this investigation was:

1. To determine the effect of subclinical respiratory infection with Cryptococcus neoformans on the host defense mechanism, when this host was exposed to a lethal challenge with the organism.

2. To elucidate the pathogenesis of Cryptococcus neoformans and its rate of dissemination to different organs of infected animals following respiratory infection.

3. To determine the extent of phagocytosis and the intracellular fate of C. neoformans by alveolar and peritoneal macrophages isolated from cryptococcal infected animals during different stages of infection.

CHAPTER II

MATERIALS AND METHODS

Respiratory Exposure of Mice to Cryptococcus neoformans. The Henderson Apparatus, a modification of that apparatus described by Henderson in 1952 (24) was used to expose the mice to aerosols generated by a collison nebulizer. The total, non-circulating air flow through the apparatus was 8 liters/minute of airborn particles of 10 microns or less. The cloud was assayed by sampling with porton all glass short impingers (12 liters/minute) that contained 10 ml of nutrient buffered gelatin. This medium consisted of 2 g gelatin, 4 g Na_2 HPO_4 anhydrous, and 37 g Brain Heart infusion (Difco) dissolved in one liter of distilled water, pH = 7.0 $\pm$ 0.2, to which had been added 4 drops of sterile olive oil (3). The aerosol sampling period was two minutes in all experiments. The animals were exposed for 10 minutes followed by one minute sterile air wash. The ambient air temperature in the exposure chamber was 76 - 86° F. and the relative humidity was maintained at 82%.

The formulae for determination of spray factor and infective dose were those elaborated by Elberg and Henderson, 1948 (16). Three impingers were used to sample the aerosol before and after each exposure period. Quantitation of viable cells/ml was done by plating 0.1 ml

aliquots of dilution of impinger fluid on Sabouraud's Dextrose Agar plates with 100 units/ml of penicillin and 100 mg/ml of streptomycin. Samples from the nebulizer which contained physiological sterile saline (PSS) as a suspending fluid also were plated for quantitation of viable cells in the same manner as the impinger fluid. The concentration of cells in the nebulizer was 3.3×10^8 and the infecting dose was 1.6×10^4 viable cells per mouse.

Experimental Animals. Female inbred BALB/cj mice, six to eight weeks of age and weighing approximately 25 - 30 grams were the experimental animals used throughout the investigation. The animals were housed 10 per cage on autoclaved litter and received water and mouse chow ad libitum.

Organisms. Cryptococcus neoformans (isolate 184), a weakly encapsulated yeast, was used for infection and challenge. The culture was originally obtained from a patient at Charity Hospital in New Orleans, Louisiana by Dr. Lorraine Friedman. In 1961, our laboratory obtained this weakly encapsulated isolate and has maintained it since on Sabouraud's Dextrose Agar.

Preparation of Cryptococcus neoformans Yeast Cell Suspensions. Yeast cells of C. neoformans 184 were grown on Sabouraud's Dextrose Agar slants for 72 hrs at 30° C. The cells were washed from the agar slants with physiological sterile saline (PSS, 0.85% NaCl). The suspension was centrifuged at 200 x g for 10 min at 4° C, and the resulting sediments were washed three times, counted by a hemacytometer and then resuspended and diluted in PSS to the concentrations which were

used in infection, challenge, LD_{50} determination and in the in vitro phagocytic studies. Viability of C. neoformans was determined as in the LD_{50} studies.

Tissue Cultures. Hanks Balanced Salt Solution (HBSS) containing 5 units heparin, 100 units/ml of penicillin and 100 mg/ml of streptomycin was used to collect the alveolar and peritoneal macrophages. Eagle's minimal essential medium (MEM) containing 20% fetal calf serum, 1% L-glutamine, 100 units/ml of penicillin and 100 mg/ml of streptomycin was used to maintain the macrophage cultures.

Determination of LD_{50} for Intravenous Challenge with Cryptococcus neoformans. The inoculum for LD_{50} determination was yeast cells prepared as before. A suspension of C. neoformans yeast cells containing 1.7×10^8 cells/ml was serially diluted to determine viability of the organism. The LD_{50} studies consisted of eleven groups of ten mice each. Animals in ten of the groups were inoculated intravenously in the tail vein with 0.1 ml of yeast cells in concentrations ranging from 1.7×10^1 to 1.7×10^8. One group was used as control and each animal was innoculated IV with 0.1 ml PSS. Deaths were recorded for each dilution as the number dead/total each day for 21 days. LD_{50} was calculated according to the method of Reed and Meunch (52). The 21 day LD_{50} was determined to be 8.5×10^5 viable C. neoformans cells/ml. Two LD_{50} (1.7×10^6 C. neoformans cells/ml) were used to challenge the animals in the mortality studies.

Mortality Studies. To determine the effect of subclinical infection with Cryptococcus neoformans on the resistance of mice to a

lethal challenge with the same organism, 9 groups of 10 mice each were infected with 1.6×10^4 viable yeast cells. The infected animals of each group were then challenged intravenously with $2LD_{50}$ (1.7×10^6) of viable C. neoformans/ml at different days (i.e. 1, 3, 7, 14, 21, 28, 42, 56, and 84 days) following primary aerosol exposure. Fifty normal mice were challenged with $2LD_{50}$ and used as a challenge control.

Mice were observed daily until the experiment was terminated. Deaths were recorded and autopsies were performed to confirm the cause of death. Enhanced immunity against challenge with C. neoformans was determined both by lower rate of mortality and prolongation time of survival.

Pathogenesis Studies. The enumeration of viable infecting C. neoformans cells in different organs of mice was used to follow the pathogenesis of the organism and its rate of dissemination as a result of activated or suppressed cellular immune response. Six mice were autopsied on days 1, 3, 7, 14, 21, 28, 42, 56, and 84 post exposure to aerosolized C. neoformans. Lungs, livers, spleens and brains were removed aseptically from each mouse and homogenized in tissue grinder tubes (A. H. Thomas Co., Philadelphia, Pa.) containing 9 ml of PSS. One ml of organ homogenate from each grinder tube was diluted serially and plated in triplicate on Sabouraud's dextrose agar plates containing 100 units/ml of penicillin and 100 mg/ml of streptomycin. All plates were quantitated after incubation for three days at 30° C by enumeration of viable C. neoformans colonies from each organ homogenate.

In vitro Phagocytic Studies. To study the role of macrophages in immunity and pathogenesis of C. neoformans, alveolar and peritoneal macrophages to be used in the in vitro phagocytic studies were isolated from the same groups of animals used in pathogenesis studies which were autopsied on days 1, 3, 7, 14, 21, 28, 42, 56, and 84 post exposure. Immune peritoneal and alveolar macrophages were isolated from immune mice (i.e. mice infected with 1.6×10^4 C. neoformans and had survived the lethal challenge with 1.7×10^6 viable C. neoformans) The macrophage monolayers were parasitized in vitro with C. neoformans or Histoplasma capsulatum to determine if activated macrophages could display both immune specific and nonspecific activity against the homologous and heterologous organisms.

1. Isolation of alveolar macrophages. Alveolar macrophages were collected by a modification of the method described by Russell and Roser (57). The skin was cut over the neck area, the pretracheal muscles were dissected away and the trachea was cleaned from connected tissues. An aperture was made in the trachea below the larynx with 26 G needle, and a plastic canula connected to a 22 G needle was inserted into the trachea through the aperture and was tightly tied to the trachea by serum and moisture proof silk threads obtained from Ethicon Inc. Somerville, N.J. One ml of Hank's balanced salt solution (HBSS) containing 5 units of heparin, 100 units/ml of penicillin and 100 mg/ml of streptomycin was injected into the lung from a 1 ml syringe. After a few seconds the washing fluid was withdrawn into the syringe, injected again into the lungs and withdrawn without massaging

the lungs. The washing was repeated three times. The lung lavage was centrifuged at 200 x g for 10 min at 4° C and the supernatant was discarded. All pellets were washed three times with HBSS without heparin and adjusted with MEM to an appropriate concentration.

2. Isolation of peritoneal macrophages. Peritoneal macrophages were obtained from infected and normal mice without the use of an exudate inducing agent. A small ventral midline incision was made in the peritoneal wall. The viscera and peritoneal cavity were washed twice with 4 ml of HBSS containing 5 units of heparin/ml, 100 units/ml penicillin, and 100 mg/ml of streptomycin. The peritoneal washing was followed by centrifuging at 200 g at 4° C for 10 min and the supernatant was discarded. All pellets were washed three times with HBSS without heparin and adjusted with MEM to an appropriate concentration. Differential cell counts were done on both alveolar and peritoneal cell suspensions using Wright stain and viability of all cells was determined by the Trypan Blue exclusion test.

3. Experimental approach to in vitro phagocytic studies.

A. Extent of phagocytosis. A modification of the procedure reported by Mitchell and Friedman (40) was used. Alveolar and peritoneal macrophages from normal or infected mice were resuspended at a concentration of 1 x 10^5 cells/ml and 1 x 10^6 cells/ml respectively in MEM containing 20% fetal calf serum (FCS), 1% L. glutamine, 100 units/ml of penicillin and 100 mg/ml of streptomycin. The macrophage suspensions was distributed to a series of 16 x 85 mm rubber-stopped leighton tubes containing 10 x 50 mm coverslips (1 ml of macrophage suspension/tube). The tubes were incubated at 37° C for 4 hrs to allow attachment

of macrophages to coverslips. The nonattached cells were discarded by replacing the medium with fresh MEM and the cell cultures were reincubated for 24 hrs. Cryptococcus neoformans were washed with MEM from the surface of Sabouraud's dextrose agar slants after growing for 72 hrs at 30° C. Histoplasma capsulatum yeast cells were washed with MEM from the surface of brain heart infusion slants with 5% human blood after growing at 37° C for 72 hrs. Cryptococcus neoformans and H. capsulatum washings were centrifuged at 200 x g for 10 min at 4° C; the pellets were washed three times with MEM. Hemacytometer count was done and the cells were resuspended in MEM with FCS, L-glutamine and antibiotics to the appropriate concentration.

Monolayers of alveolar macrophages from infected and normal animals were parasitized in vitro with 1 x 10^6 C. neoformans or H. capsulatum, while the monolayers of peritoneal macrophages were challenged with 1 x 10^7 C. neoformans or H. capsulatum. The infective organisms were suspended in 1 ml of MEM containing 20% FCS and added to the macrophage monolayers in 1 ml quantities to give a macrophage-organism (C. neoformans or H. capsulatum) ratio of approximately 1:10. Control tubes contained only MEM. After 3 hrs of incubation at 37° C to permit phagocytosis, extracellular organisms were eliminated from all the tubes by three washes with warm MEM without FCS. At this time two coverslips were removed from two randomly selected tubes, fixed in methanol and stained with Wright's stain. The remaining tubes were replenished with warm MEM containing FCS and reincubated for 24 and 48 hrs, at which time two coverslips were removed and stained. Two hundred macrophages

were examined per coverslip. Both the percentage of macrophages that contained intracellular yeast and the total number of intracellular yeasts were determined.

B. Intracellular fate of Cryptococcus neoformans. A modification of the procedure reported by Simmons and Kornovsky (58) was used for disruption of macrophages to determine the quantitative recovery of viable intracellular C. neoformans cells. Two coverslips from two corresponding tubes were transferred to sterile leighton tubes which were chilled on ice. The coverslips were washed three times with MEM and then 1 ml portions of sterile 0.5% Triton X-100 (Rohm and Haas Co., Philadelphia, Pa.) in 85% NaCl were added to each tube. The monolayers were then suspended and lysed by rapid pipetting with sterile pasteur pipets and 0.1 ml samples were serially diluted for plating in sterile 0.85% NaCl. Treatment of macrophage monolayers with this concentration of Triton X-100 did not affect the viability of Cryptococcus neoformans whereas it promoted the release of macrophages from coverslips and resulted in 90 - 95% lysis of macrophages as revealed by microscopic examination of the treated monolayers. The viability of C. neoformans in the Triton containing medium was determined in each experiment. After the macrophage monolayers were lysed and diluted, the samples were plated on Sabouraud's dextrose agar plates containing 100 unit/ml of penicillin and 100 mg/ml of streptomycin. The plates were incubated at 30° C and viable organisms were counted after 72 hrs of incubation.

Statistical Analysis. Means, standard error, and unpaired t. test programmed on the Hewlett-Packard calculator model 9810A were used in analysis of data.

CHAPTER III

RESULTS

Pathogenesis Studies

Groups of six Balb/cj infected mice were sacrificed at days 1, 3, 7, 14, 21, 28, 42, 56, 84 post respiratory infection with 1.6×10^4 viable C. neoformans. The numbers of organisms in the lungs, livers, spleens, and brains were quantitated for each mouse. The mean number of viable C. neoformans isolated from different organ homogenates for each sample group was determined by the sum total number of organisms recovered from infected mice in each group divided by the total number of mice in that group.

The results in Table I indicate that the lungs were the primary site of infection with C. neoformans. However, infection was not established in the lungs until the seventh day following respiratory exposure. The organism remained in the lungs at least 14 days post infection. This observation suggests that C. neoformans may initially colonize certain foci in the upper respiratory tract such as mucous membranes or lymphoid tissues, prior to further multiplication and dissemination, which leads to the establishment of a primary focus of infection in the alveoli of the lungs. Pulmonary cryptococcal

Table 1

Percentage of isolation of Cryptococcus neoformans from organs of mice exposed to 1.6×10^4 viable yeast cells.

Days post Exposure	Lung	Liver	Spleen	Brain
1	0	0	0	0
3	0	0	0	0
7	83.33	0	0	0
14	83.33	0	0	0
21	100	33.33	0	0
28	100	33.33	0	0
42	100	83.33	33.33	33.33
56	100	16.66 *	16.66 *	33.33 **
84	100	33.33	33.33	33.33
Control	0	0	0	0

* Mice had no dissemination of C. neoformans to the brain.

** Mice had cleared spleens and livers but lungs and brains were positive to C. neoformans.

infection appears to provide the primary source of yeast for subsequent dissemination to other organs later in the infective process. These results indicate that the pathway of dissemination of cryptococcosis is from the primary pulmonary infection to the liver, spleen, and finally to the brain in selected animals.

Animals sacrificed at days 7 and 14 post infection showed insignificant increase in number of viable C. neoformans in the lungs (Fig. 1), eventhough 83% of the infected animals were found to have viable yeast in their lungs (Table 1). The data in Table I shows that dissemination of C. neoformans to the liver started at day 21 post infection and the organism was restricted to this organ until day 28 post infection with very low rate of fungus multiplication (Fig. 2). Dissemination of the organism to the spleen and brain was not observed until day 42 post infection (Fig. 3, Fig. 4). At this time, there was a progressive increase in number of viable C. neoformans in both lungs and livers (Fig. 1, Fig. 2). On day 56 post infection, the lungs were 100% positive to C. neoformans with rapid proliferation of the organism in this organ. The livers and spleens were positive only in 16.66% of these animals with no dissemination to the brain (Table 1). The livers and spleens of the remaining mice in the same group were cleared from infection with C. neoformans but 33.33% of these animals had dissemination to the brain. This unusual course of dissemination resulted in a remarkable decrease in the number of viable C. neoformans recovered from liver and spleen homogenates (Fig. 2, Fig. 3), and a corresponding increase in the number recovered from brain homogenates (Fig. 4). On

Figure 1. Quantitative assay of viable Cryptococcus neoformans from lungs isolated from infected mice at different intervals post respiratory infection with C. neoformans.

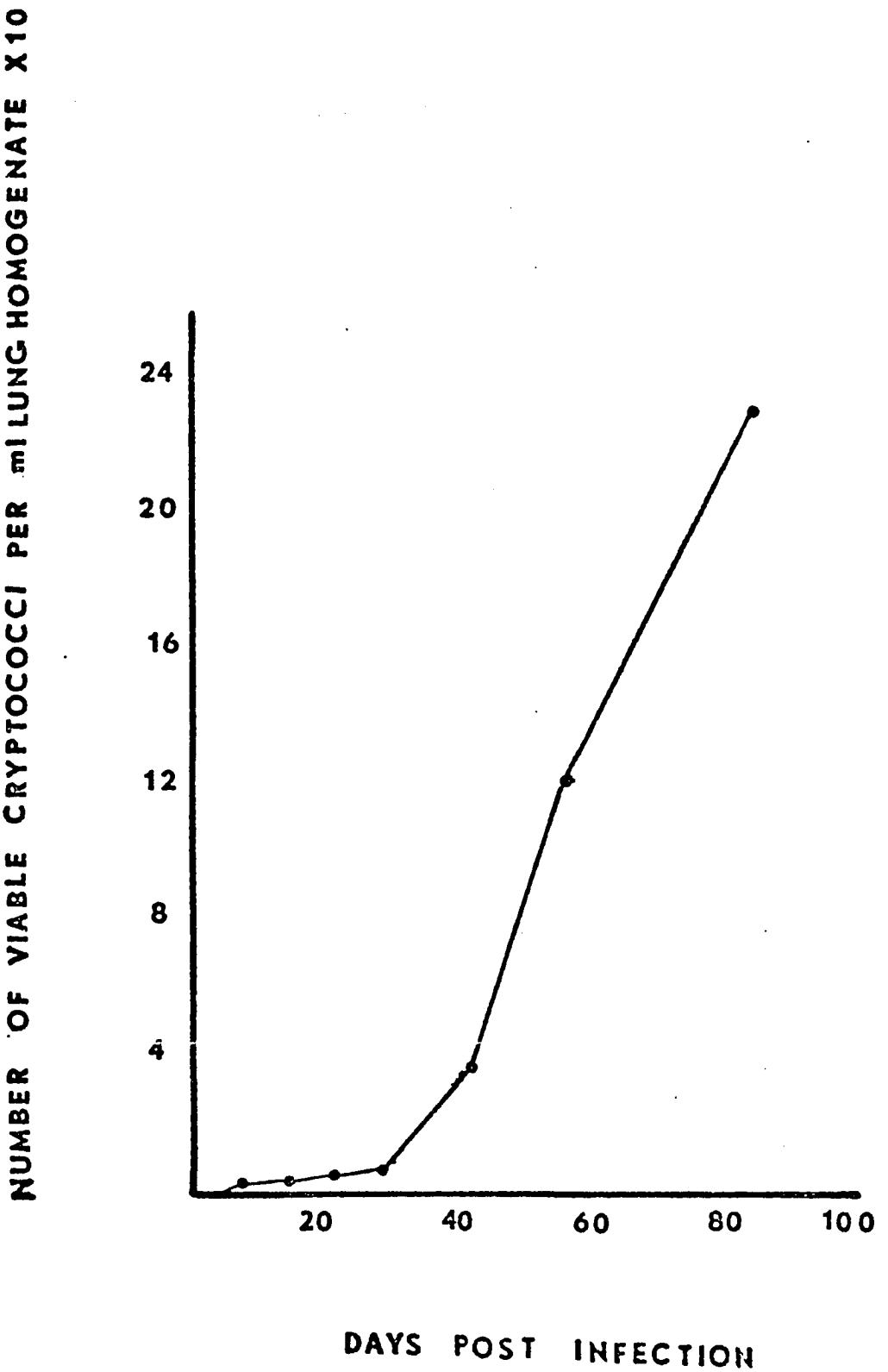

NUMBER OF VIABLE CRYPTOCOCCI PER ml LUNG HOMOGENATE X10 4
24
20
16
12
8
4
20
40
60
80
100
DAYS POST INFECTION

Figure 2. Quantitative assay of viable Cryptoccus neoformans from livers isolated from infected mice at different intervals post respiratory infection with C. neoformans.

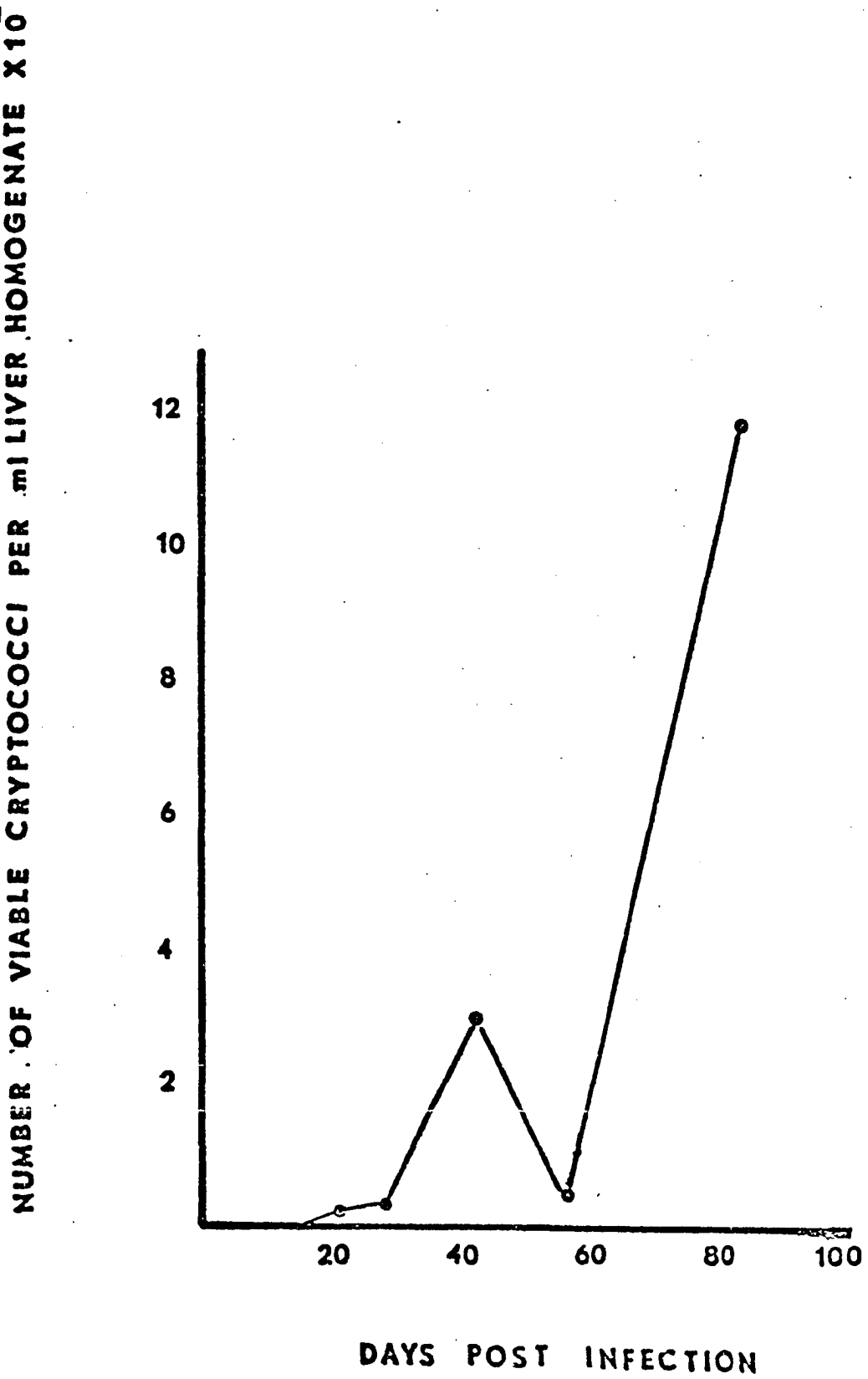

NUMBER OF VIABLE CRYPTOCOCCI PER ml LIVER HOMOGENATE X10 2
12
10
8
6
4
2
20
40
60
80
100
DAYS POST INFECTION

Figure 3. Quantitative assay of viable Cryptococcus neoformans from spleens isolated from infected mice at different intervals post respiratory infection with C. neoformans.

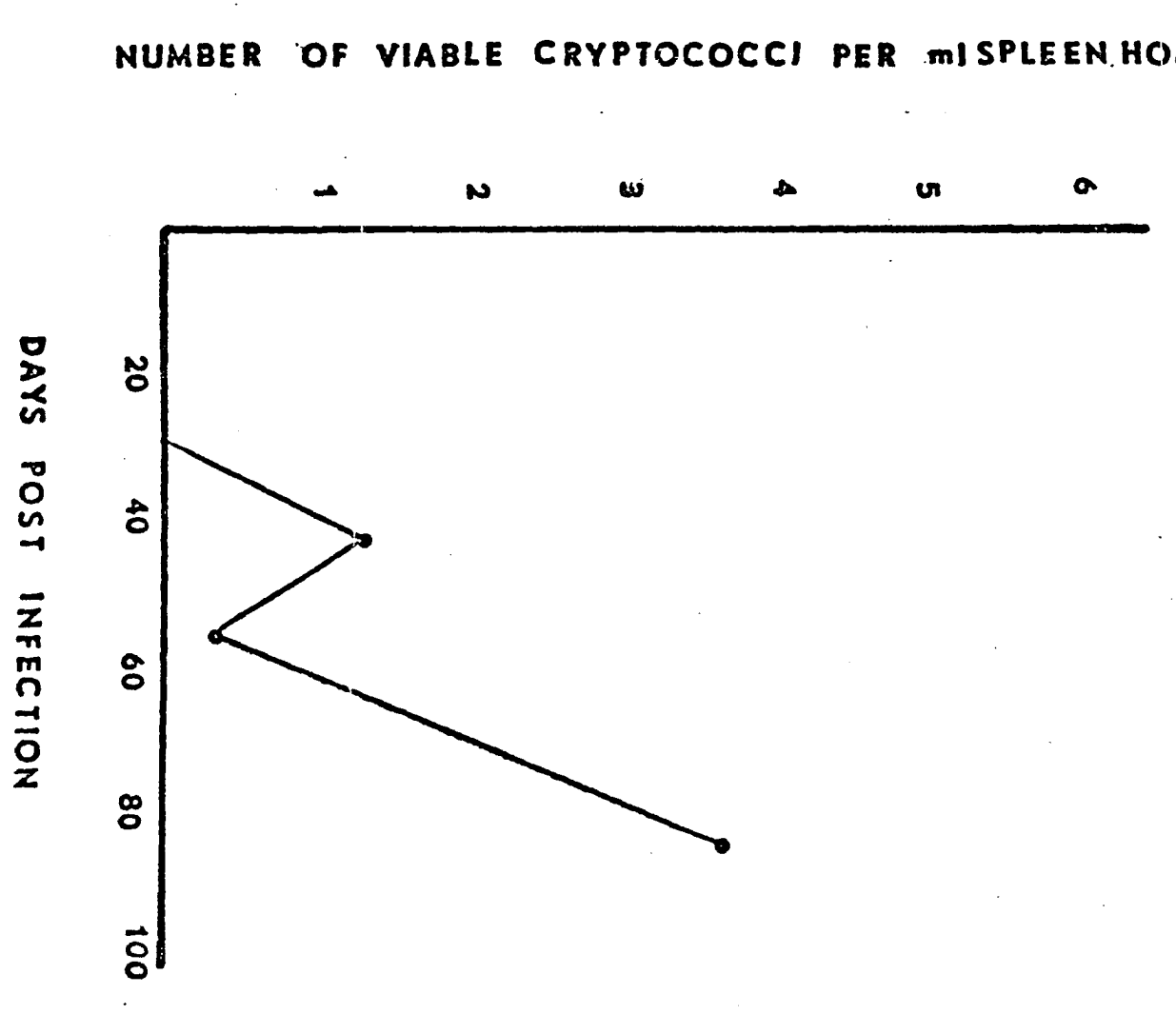
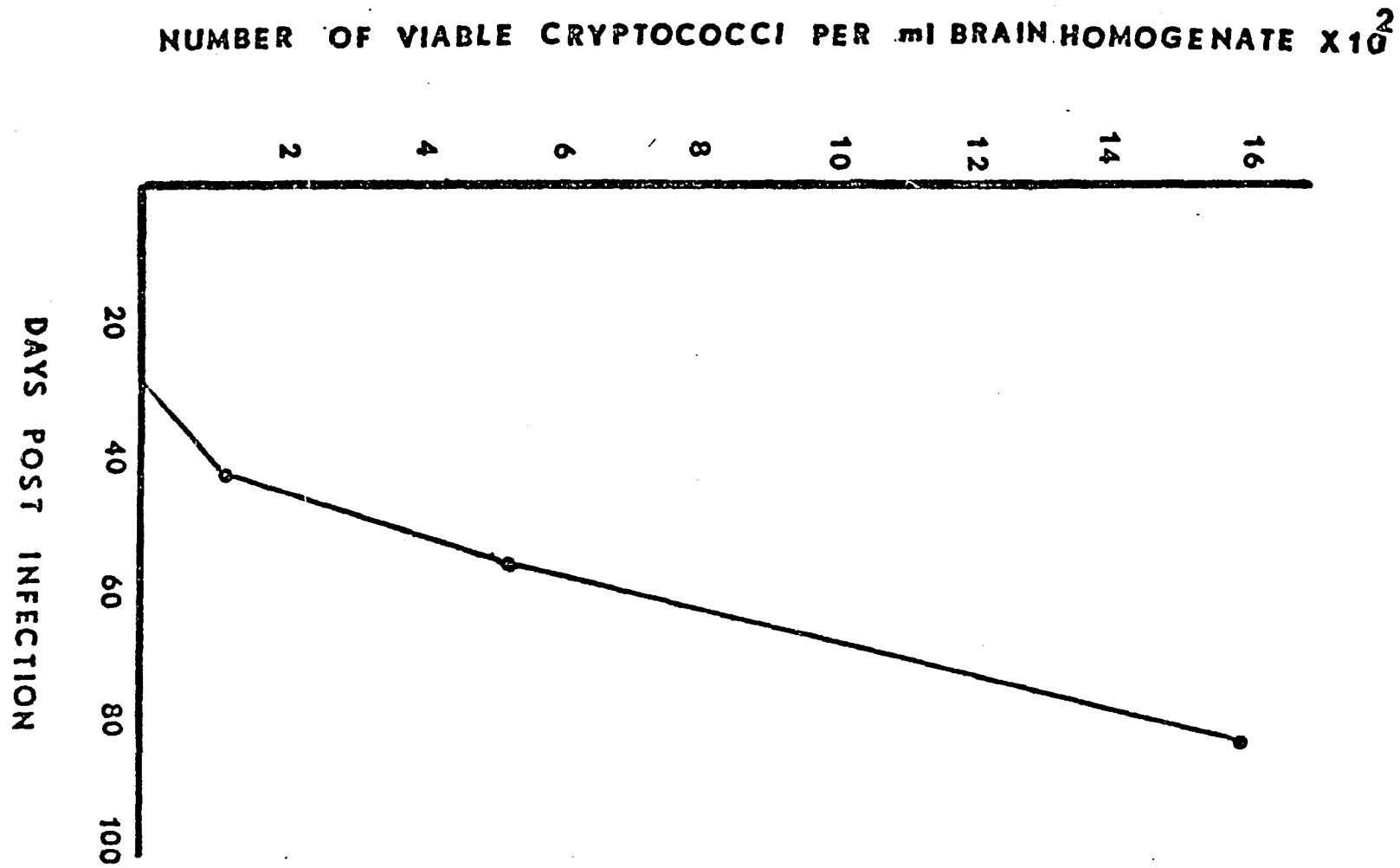

NUMBER OF VIABLE CRYPTOCOCCI PER ml SPLEEN HOMOGENATE X10²
1
2
3
4
5
6
DAYS POST INFECTION
20
40
60
80
100

Figure 4. Quantitative assay of viable Cryptococcus neoformans from brains isolated from infected mice at different intervals post respiratory infection with C. neoformans.


NUMBER OF VIABLE CRYPTOCOCCI PER ml BRAIN HOMOGENATE X10²
2
4
6
8
10
12
14
16
DAYS POST INFECTION
20
40
60
80
100

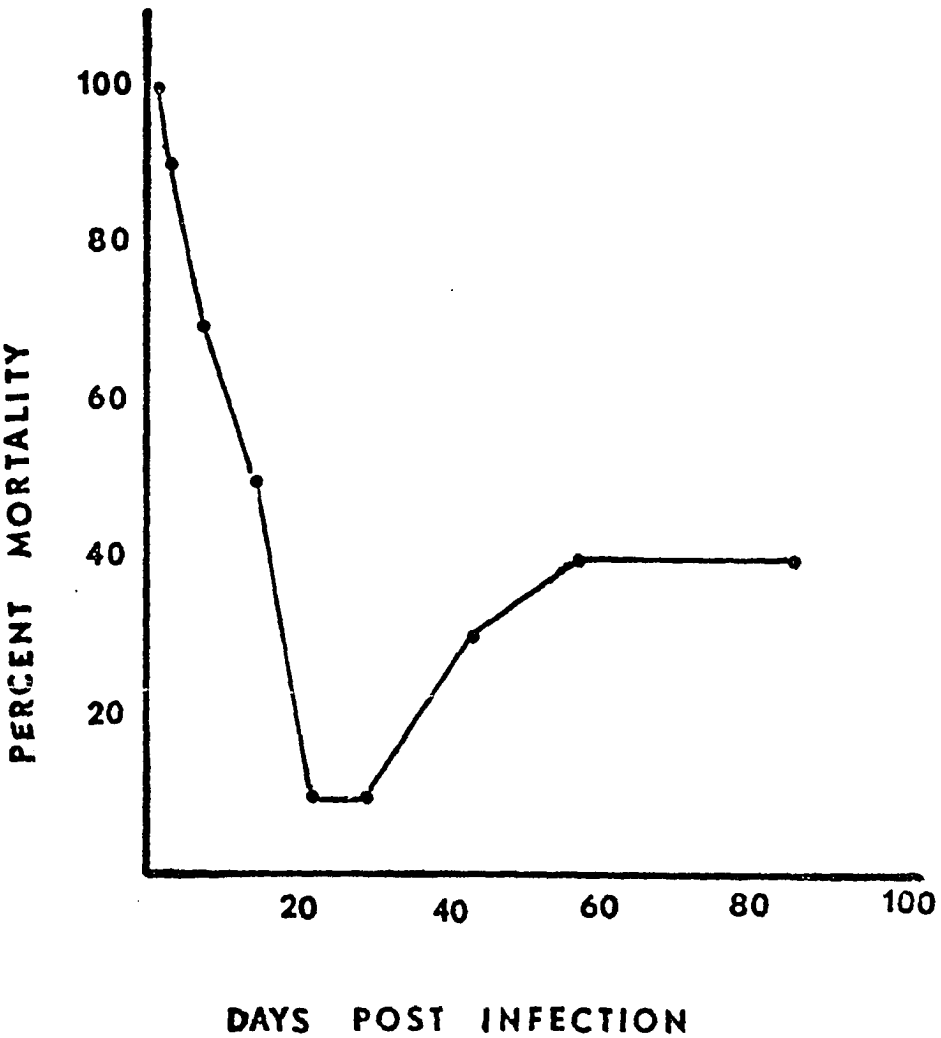
day 84 post infection, the same pattern of organism proliferation was observed in lungs and brains (Fig. 1, Fig. 4), but in contrast, C. neoformans was isolated from livers and spleens in 33.3% of the animals after a temporary period of clearance which had been observed 56 days post infection (Table 1). The number of viable C. neoformans was also significantly increased at day 84 post infection as seen in Fig. 2 and Fig. 3.

Normal controls consisted of groups of 3 noninfected Balb/cj mice which were sacrificed at each time period. All organ homogenates from these animals were culturally negative to C. neoformans at all time periods studied.

Mortality Studies. Groups of 10 mice sublethally infected by the aerosol route with 1.6×10^4 viable yeast cells were challenged intravenously with 1.7×10^6 ($2LD_{50}$) viable C. neoformans at days 1, 3, 7, 14, 21, 28, 42, 56, and 84 post infection. Mice were observed daily and deaths were recorded. Enhanced immunity against lethal challenge with C. neoformans was determined by lower rate of mortality and prolongation time of survival. The results in Fig. 5 show a high rate of mortality at day 1 post infection which progressively decreased as immunity increased until day 28 post infection. However, a curious reversal in immune protection occurred as the rate of mortality began to increase again at day 42 post infection and continued to increase until day 56 when it was maintained at the same level until day 84 post infection.

The survival time varied from one group to another. Animals

Figure 5. Cumulative 3 weeks mortalities of 10 mice challenged intravenously with 1.7×10^6 viable C. neoformans at different intervals post respiratory infection with the organism.


100
80
60
40
20
PERCENT MORTALITY
20
40
60
80
100
DAYS POST INFECTION

challenged at days 1 and 3 post infection showed 100% and 90% death at the end of observation period which was three weeks post challenge. Noninfected control mice reached 100% mortality during the same observation period. Animals challenged on days 7, 14 post infection showed 70% and 50% mortality three weeks post challenge; and the surviving animals lasted one week longer and then died. Animals challenged at days 21 and 28 post infection showed 10% mortality three weeks post challenge. The surviving animals from day 21 survived two weeks longer and then all succumbed. In contrast, the surviving animals from day 28 survived until the end of the experiment with a very low mortality rate; but all died by 77 days post challenge. Animals challenged on days 42, 56, 84 post infection showed 30%, 40%, and 40% mortality respectively three weeks post challenge. However, animals from the three groups survived only one week longer.

All challenged, noninfected control mice showed 100% mortality at the end of observation period which was three weeks post challenge.

Isolation of Macrophages. The isolation techniques provided an adequate yield of alveolar (AM) and peritoneal macrophage (PM). The cell yields increased with the progression of cryptococcal infection. Microscopic examination of Wright's stained smears from the peritoneum and lung exudates of normal and infected mice showed approximately 90% of the cells were macrophages, 7% lymphocytes, and 3% polymorphonuclear cells.

Trypan Blue exclusion test for alveolar and peritoneal macrophages from infected and noninfected animals revealed a viability of

90 - 95%.

In vitro Phagocytic Studies. The events of phagocytosis of C. neoformans by alveolar and peritoneal macrophages isolated from infected mice during different stages of progressive cryptococcosis, was assessed in vitro. Also, the fungicidal or fungistatic activity of alveolar and peritoneal macrophages were evaluated with regard to their capacity to kill intracellular C. neoformans or inhibit intracellular multiplication. All macrophage monolayers from infected and noninfected animals were parasitized in vitro with C. neoformans. Monolayers of immune macrophages (i.e. peritoneal and alveolar macrophages isolated from mice infected with 1.6×10^4 C. neoformans and had survived the lethal challenge with 1.7×10^6 viable C. neoformans) were parasitized in vitro with Cryptococcus neoformans or Histoplasma capsulatum. The in vitro infection of macrophage monolayers with these two organisms was aimed to determine if activated macrophages could display both specific and nonspecific immune activity against the homologous and heterologous organisms. Viable plate counts on macrophage lysates taken at different time intervals post in vitro infection of monolayers with C. neoformans or H. capsulatum, were performed to assess the extent of fungicidal activity or intracellular multiplication of either organism.

Functional Activity of Alveolar Macrophages. The data in Table II summarize the results of the phagocytic activity displayed by alveolar macrophages. These macrophages showed no phagocytic activity at day 1 and 3 post respiratory exposure to C. neoformans. However, alveolar macrophages collected at day 7 after infection displayed

a significant rate of phagocytosis of 15% ($0.005 < P < 0.001$) which increased to 17% ($0.005 < P < 0.001$) at day 14, and then dissipated at day 21 to 7% ($0.025 < P < 0.01$) and at day 28 to 5% phagocytosis ($0.025 < P < 0.01$. On day 42 post infection there was an 8% increase ($0.005 < P < 0.001$) in the phagocytic activity of alveolar macrophages. This increase, however, does not accurately reflect increased phagocytic activity since it was accompanied by increased multiplication of the organism within the macrophages. A marked increase in the number of viable intracellular C. neoformans was observed when determination were made by viable plate counts of lysates from these disrupted macrophages (Fig. 8). Further evidence from the microscopic observation of the disintegrated infected monolayers indicated that the increased numbers of intracellular viable yeast resulted from impaired fungicidal activity rather than increased phagocytic activity of the alveolar cells. Complete impairment of phagocytic activity by alveolar macrophages occurred at day 56 and continued until day 84 post infection. Alveolar macrophages isolated from noninfected control animals, also were totally ineffective in phagocytizing C. neoformans.

Alveolar macrophages isolated from infected animals on days 7, 21, 28 and 42 post infection provided a favorable environment for the intracellular growth of C. neoformans. However, the rate of intracellular multiplication was very low within macrophages isolated on days 7 and 14 (Fig. 6), but much higher within those macrophages isolated on days 21, 28, and 42 (Fig. 7, Fig. 8). Alveolar macrophages isolated from infected mice on day 14 post infection displayed a

Table II

Extent of phagocytosis of Cryptococcus neoformans by alveolar macrophages isolated from mice at different intervals post respiratory exposure to the organism.

Sample time of phagocytosis	Percent of macrophages with ingested yeast cells (days post exposure)										Number of yeast cells per 100 macrophages (days post exposure)									
	1	3	7	14	21	28	42	56	84	Control	1	3	7	14	21	28	42	56	84	Control
3 hrs	0	0	5	8	2	0	2	0	0	0	0	0	5	9	5	0	3	0	0	0
24 hrs	0	0	13	14	6	4	3	0	0	0	0	0	19	21	9	6	5	0	0	0
48 hrs	0	0	15	17	7	5	13 *	0	0	0	0	0	23	28	12	9	39 *	0	0	0

* Disintegration of macrophage monolayers was observed.

Figure 6. Intracellular fate of C. neoformans in vitro within alveolar macrophage monolayers isolated from infected mice on days 7 and 14 post respiratory infection with C. neoformans

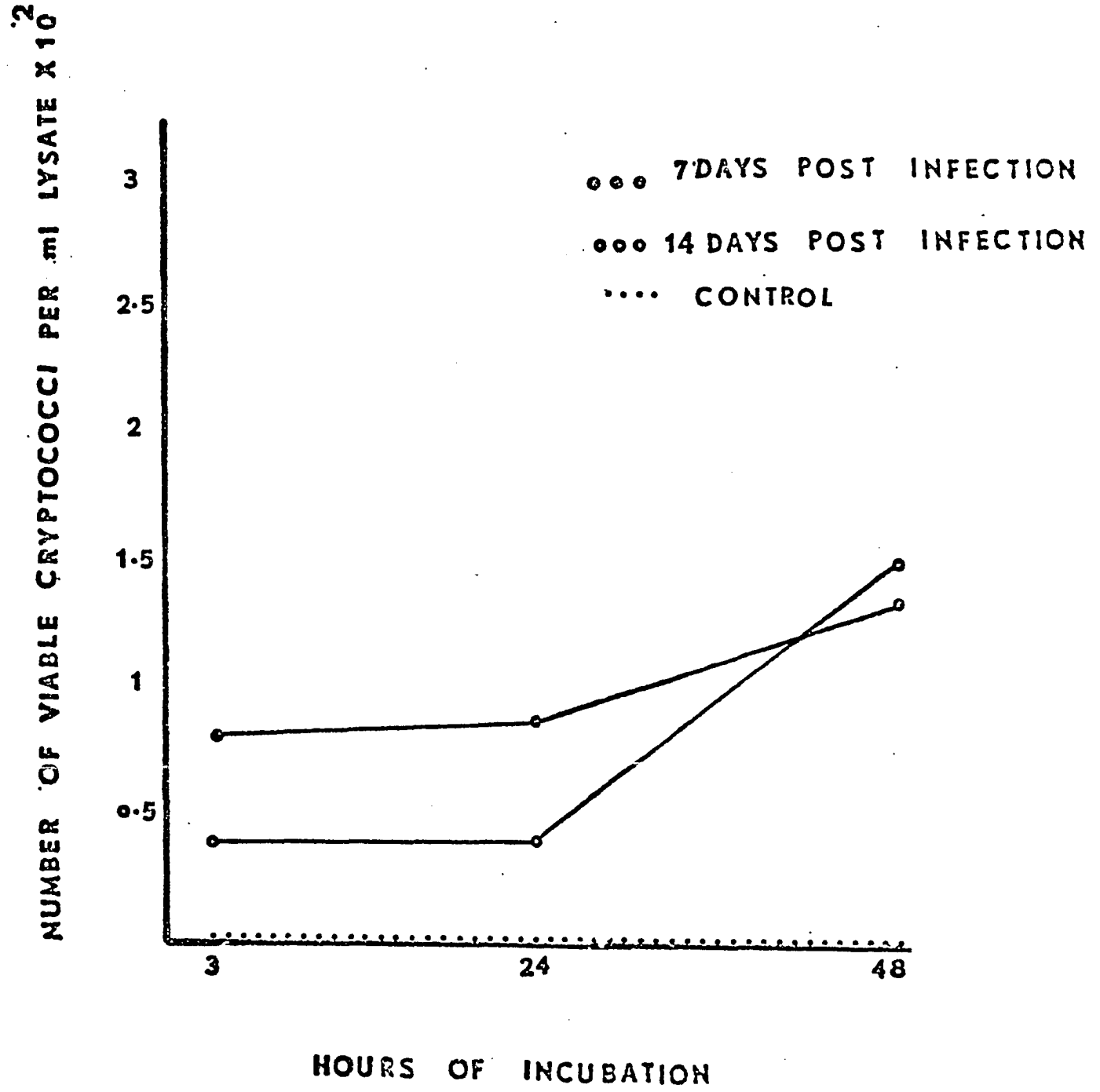

NUMBER OF VIABLE CRYPTOCOCCI PER ml LYSATE X 10^2
3
2·5
2
1·5
1
0·5
3
24
48
HOURS OF INCUBATION
7 DAYS POST INFECTION
14 DAYS POST INFECTION
CONTROL

Figure 7. Intracellular fate of C. neoformans in vitro within alveolar macrophage monolayers isolated from infected mice on days 21 and 28 post respiratory infection with C. neoformans.

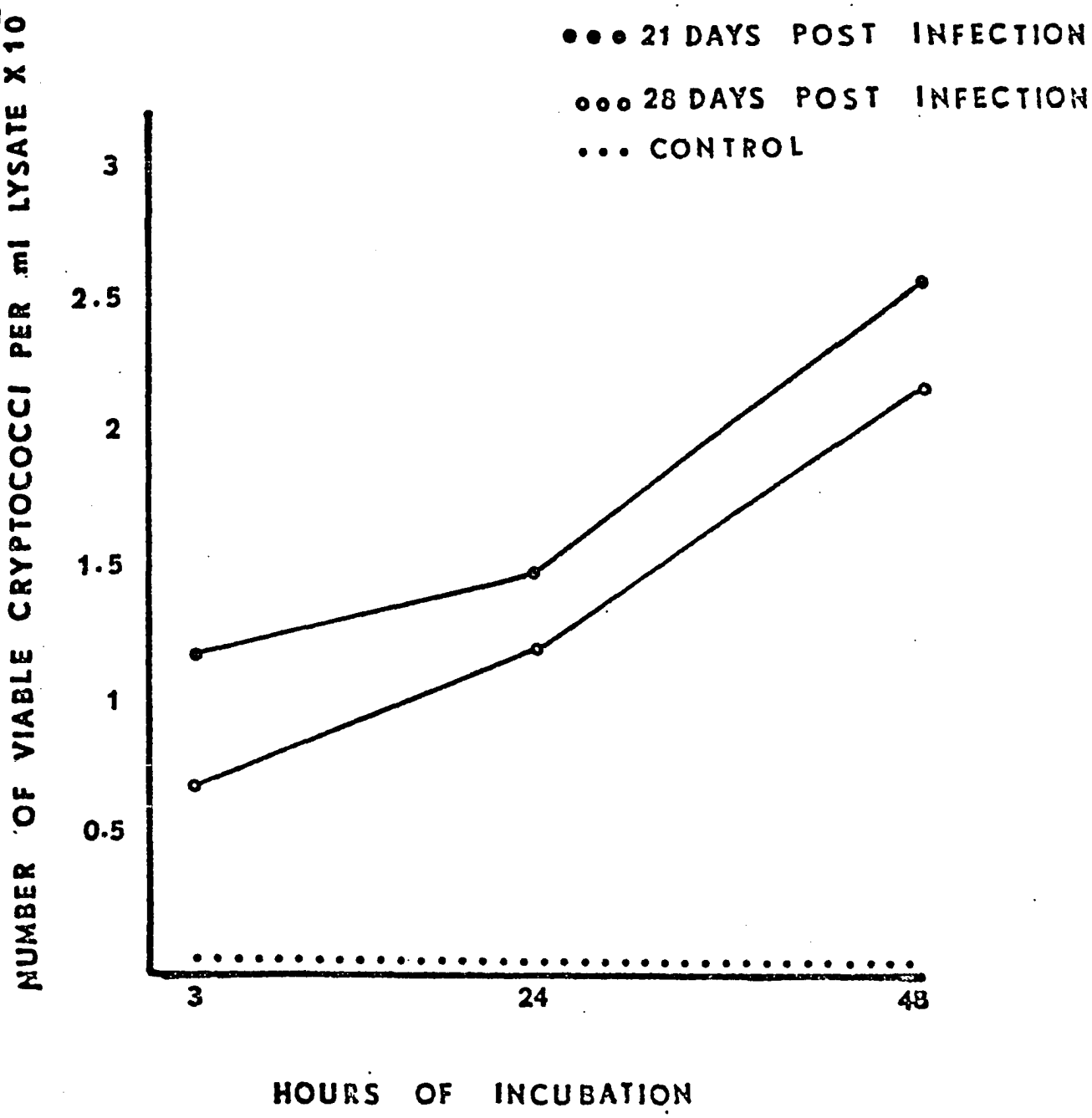

●●● 21 DAYS POST INFECTION
ooo 28 DAYS POST INFECTION
••• CONTROL
NUMBER OF VIABLE CRYPTOCOCCI PER ml LYSATE X 10²
3
2.5
2
1.5
1
0.5
3
24
48
HOURS OF INCUBATION

Figure 8. Intracellular fate of C. neoformans in vitro within alveolar macrophage monolayers isolated from infected mice on day 42 post respiratory infection with C. neoformans.

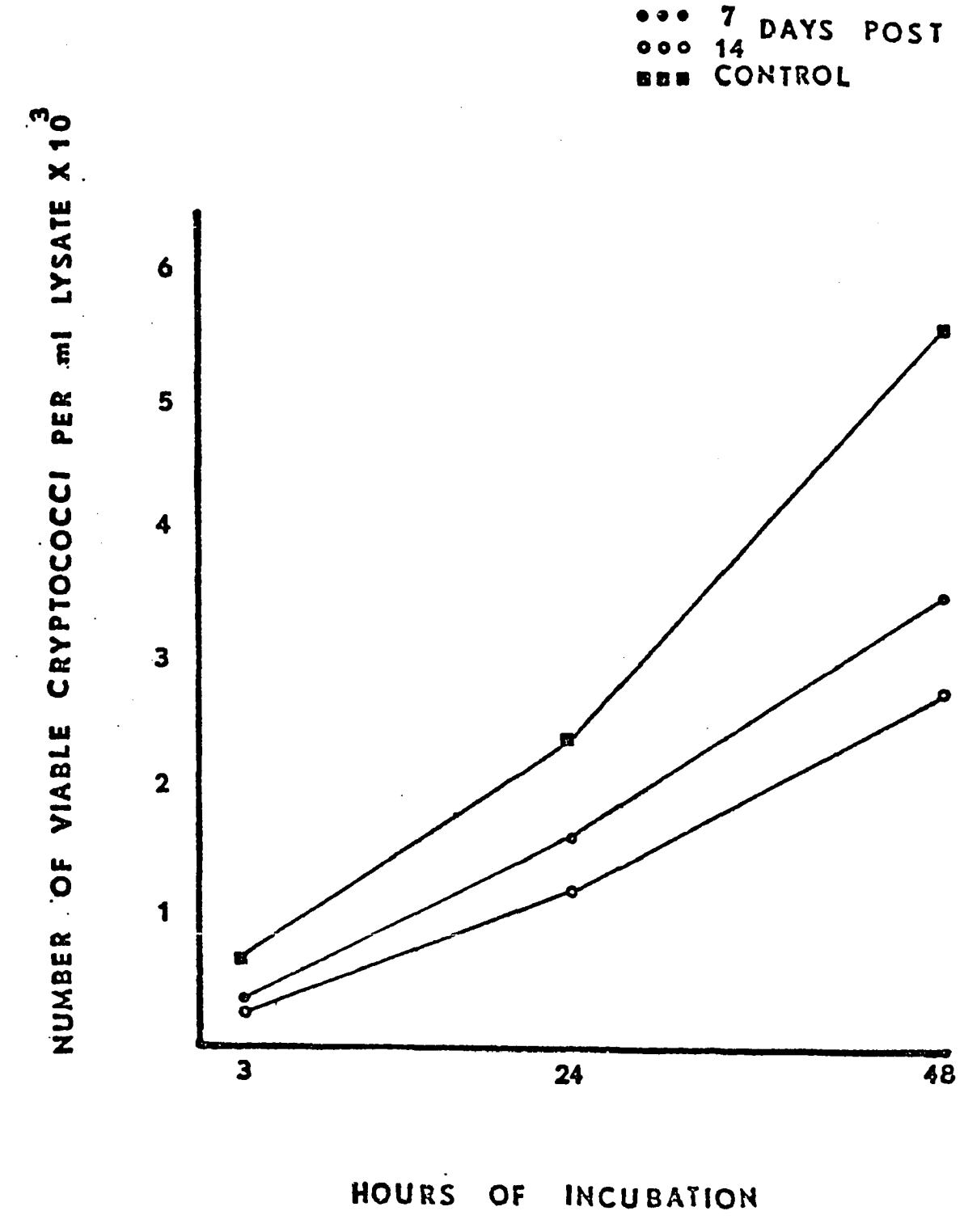

7 DAYS POST INFECTION
14
CONTROL
NUMBER OF VIABLE CRYPTOCOCCI PER ml LYSATE X 10^3
6
5
4
3
2
1
3
24
48
HOURS OF INCUBATION

Figure 11. Intracellular fate of C. neoformans in vitro within peritoneal macrophages isolated from infected mice on days 21, 28 post respiratory infection with the organism.

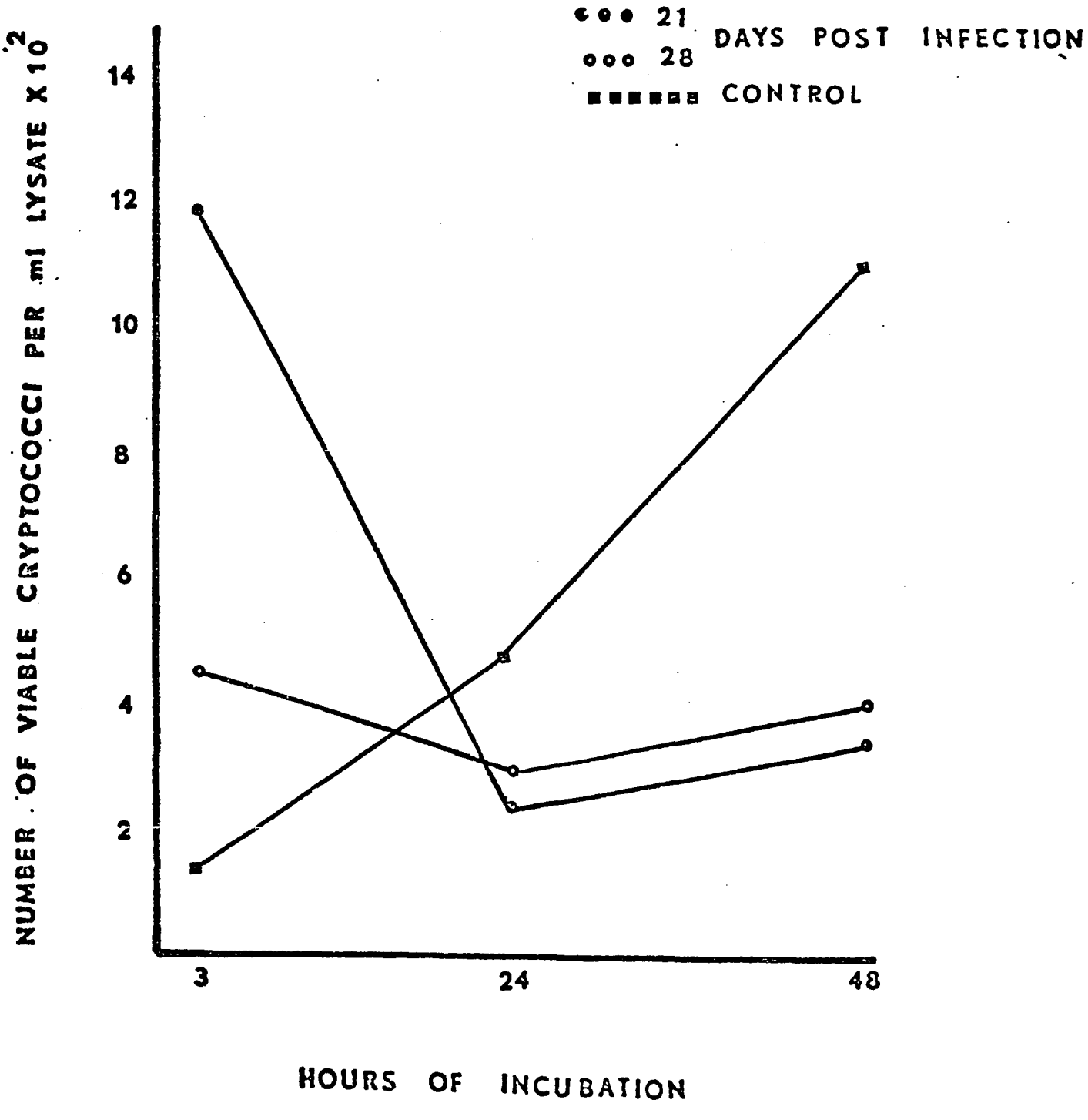
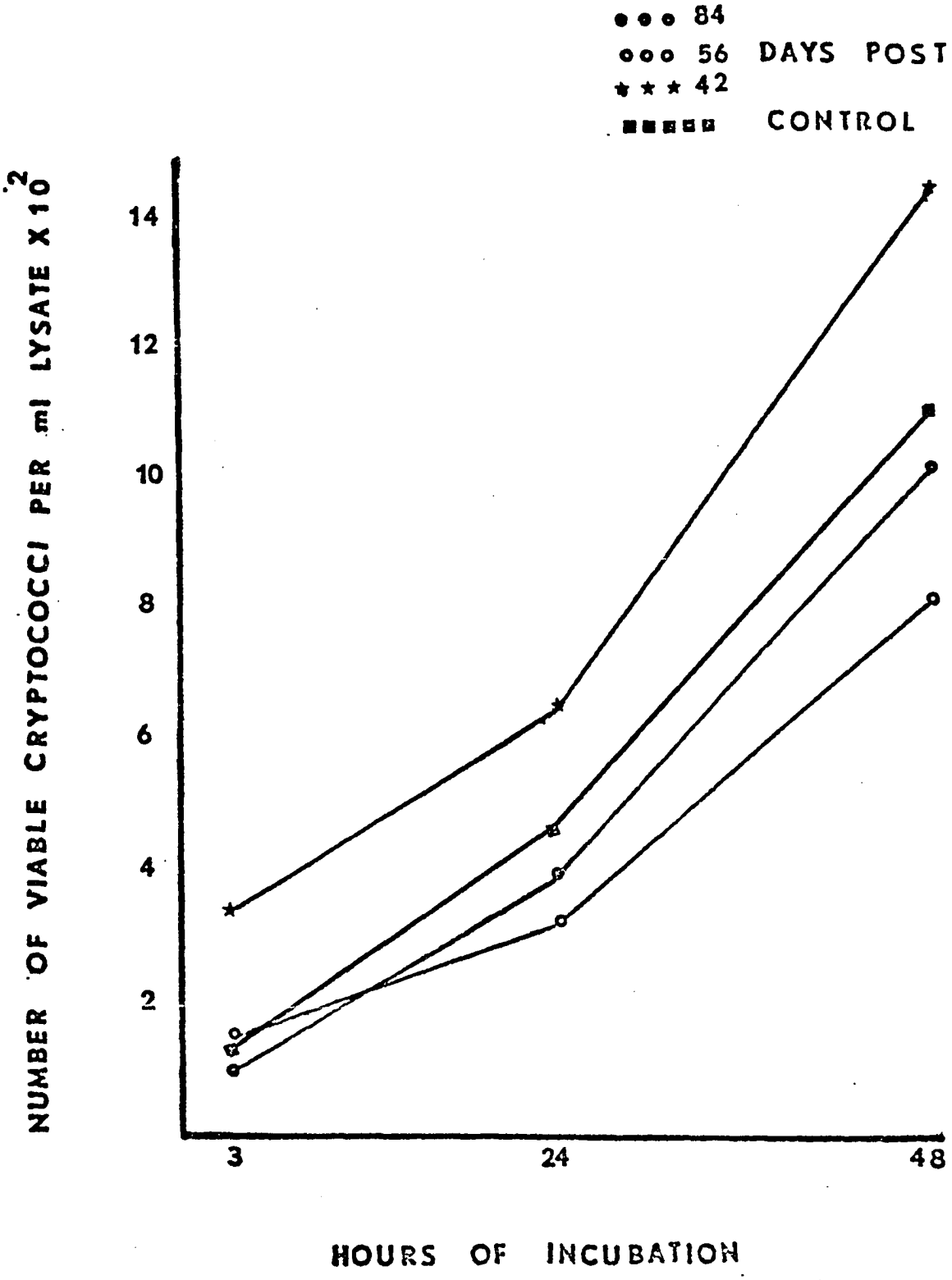

21
28
CONTROL
DAYS POST INFECTION
NUMBER OF VIABLE CRYPTOCOCCI PER ml LYSATE X 10^2
14
12
10
8
6
4
2
3
24
48
HOURS OF INCUBATION

Figure 12. Intracellular fate of C. neoformans in vitro within peritoneal macrophages isolated from infected mice on days 42, 56, 84 post respiratory infection with the organism.


● ● ● 84
○ ○ ○ 56 DAYS POST INFECTION
★ ★ ★ 42
■ ■ ■ ■ CONTROL
NUMBER OF VIABLE CRYPTOCOCCI PER ml LYSATE X 10²
14
12
10
8
6
4
2
3
24
48
HOURS OF INCUBATION

and 10% respectively ($0.05 < P < 0.025$), but without displaying any fungicidal effect on the organism as shown by the increased rate of intracellular growth of C. neoformans within these macrophages and the destruction of the macrophage monolayers after 48 hrs incubation (Fig. 12).

The results of studies on the phagocytic activity of peritoneal and alveolar macrophages obtained from immune mice (i.e. mice that were infected with 1.6×10^4 C. neoformans and had survived the lethal challenge with 1.7×10^6 viable cells of the same organism) are presented in Table IV. Peritoneal macrophages from immune donors displayed increased phagocytic activity and resistance to destruction by cryptococcal cells as compared to non-immune controls. However, both immune and normal alveolar macrophages were totally ineffective in phagocytizing the viable yeast cells. Immune peritoneal macrophages, also, showed marked fungicidal activity against C. neoformans as compared to non-immune cells (Fig. 13). These results are similar to those obtained in previous study with peritoneal macrophages assayed 21 days post primary infection (Table III, Fig. 11). Immune peritoneal macrophages from the same donors exhibited higher initial phagocytic activity by 98% to H. capsulatum which was significant at $0.1 < P < 0.05$. Such phagocytic activity, however, was not significantly higher than the phagocytic activity of normal peritoneal macrophages which produced 95% phagocytosis (Table V). On the other hand, alveolar macrophages from the same immune donors showed significant phagocytosis of H. capsulatum ($0.01 < P < 0.005$) as compared to the totally ineffective non-immune

control cells, eventhough they were not resistant to destruction by the heterologous fungus after 48 hrs of incubation (Table V). Alveolar and peritoneal macrophages from immune and noninfected control mice were found to provide a favorable environment for the early intracellular growth of H. capsulatum which led to necrosis and disintegration of the monolayers by 48 hrs post in vitro infection (Fig. 14).

Table IV

Extent of phagocytosis of Cryptococcus neoformans by peritoneal and alveolar macrophages isolated from normal and immune mice* three weeks post challenge. The immune mice were infected with 1.6×10^4 C. neoformans and challenged three weeks post infection with 1.7×10^6 C. neoformans yeast cells.

Sample time of phagocytosis	Peritoneal macrophages				Alveolar macrophages			
	Percent of macrophages w/ ingested yeast cells		Number of yeast cells per 100 macrophages		Percent of macrophages w/ ingested yeast cells		Number of yeast cells per 100 macrophages	
	Immune	Control	Immune	Control	Immune	Control	Immune	Control
3 hrs	19	7	19	8	0	0	0	0
24 hrs	30	30	45	58	0	0	0	0
48 hrs	43	30 **	60	80 **	0	0	0	0

* Infected mice that survived the lethal challenge with C. neoformans were considered immune.

** Disintegration of macrophage monolayers was observed.

Table V

Extent of phagocytosis of H. capsulatum by peritoneal and alveolar macrophages isolated from normal and immune* mice three weeks post challenge. The immune mice were infected with 1.6×10^4 C. neoformans and challenged three weeks post infection with 1.7×10^6 C. neoformans yeast cells.

Sample time of phagocytosis	Peritoneal macrophages				Alveolar macrophages			
	Percent of macrophages w/ ingested yeast cells		Number of yeast cells per 100 macrophages		Percent of macrophages w/ ingested yeast cells		Number of yeast cells per 100 macrophages	
	Immune	Control	Immune	Control	Immune	Control	Immune	Control
3 hrs	98	95	487	472	8	0	62	0
24 hrs	85 **	79 **	344	326	15	0	77	0
48 hrs	** 66	** 62	176	153	** 11	0	** 45	0

* Infected mice that survived the lethal challenge with C. neoformans were considered "immune mice".

** Disintegration of macrophage monolayers was observed.

Figure 13. Intracellular fate of C. neoformans in vitro within "immune" peritoneal macrophages isolated from infected mice 21 days post iv challenge with 1.7×10^6 C. neoformans.

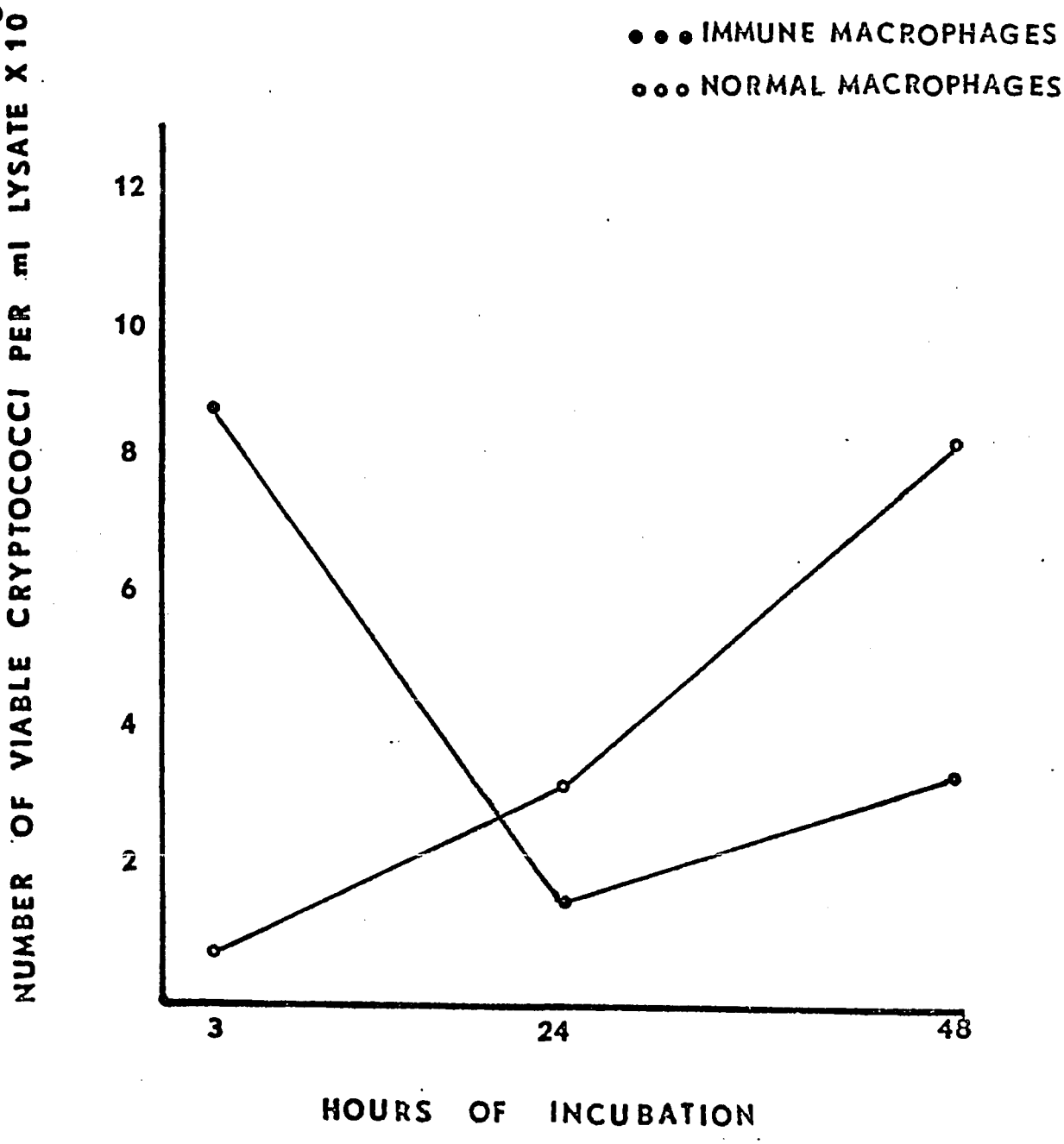

●●● IMMUNE MACROPHAGES
ooo NORMAL MACROPHAGES
NUMBER OF VIABLE CRYPTOCOCCI PER ml LYSATE X 10^3
12
10
8
6
4
2
3
24
48
HOURS OF INCUBATION

Figure 14. Intracellular fate of Histoplasma capsulatum in vitro within alveolar and peritoneal macrophages isolated from "immune mice" 21 days post iv challenge with 1.7×10^6 C. neoformans. The mice were challenged 21 days post respiratory infection with 1.6×10^4 C. neoformans.

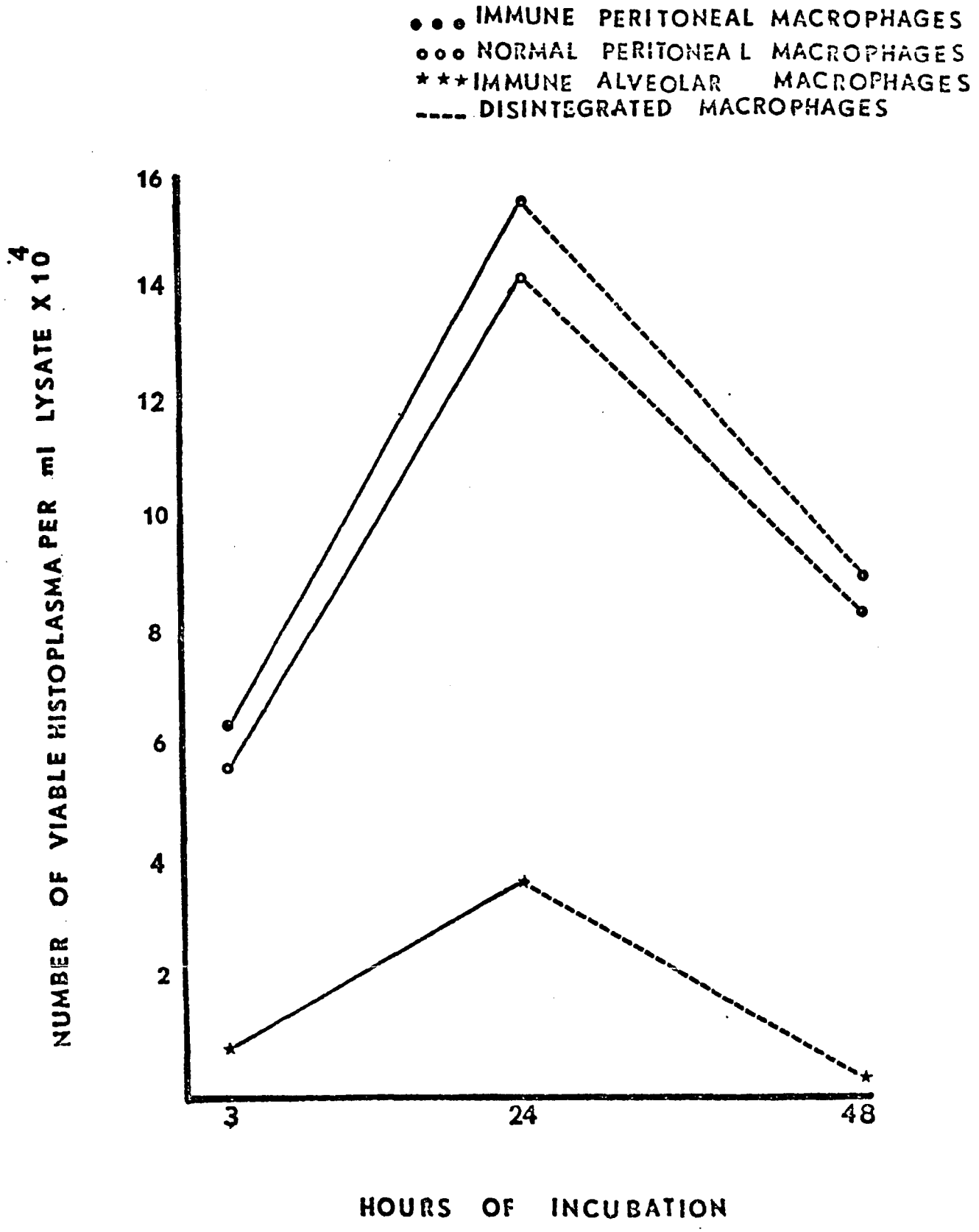

●●● IMMUNE PERITONEAL MACROPHAGES
ooo NORMAL PERITONEAL MACROPHAGES
★★★ IMMUNE ALVEOLAR MACROPHAGES
---- DISINTEGRATED MACROPHAGES
NUMBER OF VIABLE HISTOPLASMA PER ml LYSATE X 10^4
16
14
12
10
8
6
4
2
3
24
48
HOURS OF INCUBATION

Table VI. Statistical analysis comparing extent of phagocytosis of Cryptococcus neoformans by alveolar macrophages isolated from mice at different intervals post respiratory exposure to the organism.

n.s. = not significant

Days Post Exposure	t - value at different intervals of phagocytosis			p - value at different intervals of phagocytosis		
	3 hrs	24 hrs	48 hrs	3 hrs	24 hrs	48 hrs
1	0.0000	0.0000	0.0000	n.s.	n.s.	n.s.
3	0.0000	0.0000	0.0000	n.s.	n.s.	n.s.
7	5.0000	13.0000	15.0000	$0.025<P<0.01$	$0.005<P<0.001$	$0.005<P<0.001$
14	8.0000	14.0000	17.0000	$0.01<P<0.005$	$0.005<P<0.001$	$0.005<P<0.001$
21	2.0000	6.0000	7.0000	$0.1<P<0.05$	$0.025<P<0.01$	$0.025<P<0.01$
28	0.0000	4.0000	5.0000	n.s.	$0.05<P<0.25$	$0.025<P<0.01$
42	2.0000	3.0000	13.0000	$0.1<P<0.05$	$0.05<P<0.025$	$0.005<P<0.001$
56	0.0000	0.0000	0.0000	n.s.	n.s.	n.s.
84	0.0000	0.0000	0.0000	n.s.	n.s.	n.s.

Table VII. Statistical analysis comparing extent of phagocytosis of Cryptococcus neoformans by peritoneal macrophages isolated from mice at different intervals post respiratory exposure to the organism.

n.s. = not significant

Days Post Exposure	t - value at different intervals of phagocytosis			p - value at different intervals of phagocytosis		
	3 hrs	24 hrs	48 hrs	3 hrs	24 hrs	48 hrs
1	0.0000	9.3915	4.0249	n.s.	$0.01 < P < 0.005$	$0.05 < P < 0.025$
3	0.3162	5.3666	3.1305	n.s.	$0.025 < P < 0.01$	$0.05 < P < 0.025$
7	0.0000	13.4350	3.5777	n.s.	$0.005 < P < 0.001$	$0.05 < P < 0.025$
14	0.6325	6.3640	2.2361	n.s.	$0.025 < P < 0.01$	$0.1 < P < 0.05$
21	5.0596	0.5000	6.7002	$0.025 < P < 0.01$	n.s.	$0.025 < P < 0.01$
28	6.6408	0.3162	3.6056	$0.025 < P < 0.01$	n.s.	$0.05 < P < 0.025$
42	2.4962	0.0000	6.3640	$0.1 < P < 0.05$	n.s.	$0.025 < P < 0.01$
56	4.1110	4.4721	0.4472	$0.05 < P < 0.025$	$0.025 < P < .01$	n.s.
84	3.1623	2.6833	1.3416	$0.05 < P < 0.025$	$0.1 < P < 0.05$	n.s.

Table VIII. Statistical analysis comparing extent of phagocytosis of Cryptococcus neoformans and Histoplasma capsulatum by alveolar and peritoneal macrophages isolated from immune macrophages.

n.s. = not significant

Sample Time of Phagocytosis	Alveolar Macrophages				Peritoneal Macrophages			
	C. neoformans		H. capsulatum		C. neoformans		H. capsulatum	
	t. value	p. value	t. value	p. value	t. value	p. value	t. value	p. value
3 hrs	0.0000	n.s.	8.0000	$0.01 < P < 0.005$	8.4853	$0.01 < P < 0.005$	2.1213	$0.1 < P < 0.05$
24 hrs	0.0000	n.s.	15.0000	$0.005 < P < 0.001$	0.0000	n.s.	2.6833	$0.1 < P < 0.05$
48 hrs	0.0000	n.s.	11.0000	$0.005 < P < 0.001$	5.8138	$0.025 < P < 0.01$	1.4142	n.s.

CHAPTER IV

DISCUSSION

Experimental results obtained from this investigation demonstrated that sublethal respiratory infection with 1.6×10^4 C. neoformans conferred immunity on mice against a lethal challenge with 1.7×10^6 viable C. neoformans. This acquired immunity was first detected at day 7, peaked at day 21, and remained at this peak until day 28, and started to subside on day 42. An increase rate of mortality occurred at this period and continued to increase at days 56 and 84 post infection with a shorter survival time of animals challenged at these periods.

Perceval, 1965, (51) proved that animals exposed to viable C. neoformans developed more intense immunity of longer duration than the acquired immunity induced by killed cryptococci. Louria (31) suggested that the persistance of viable cells in the host offers a prolonged stimulation of host defense mechanism, while killed cells do not possess this property. Louria's observation was demonstrated in this study by the behavior of alveolar macrophages. These cells were not activated in the early stages of infection (i.e. at one and three days post exposure to C. neoformans) at which time all tissues from exposed animals were culturally negative. Activation of alveolar

macrophages occurred, however, at day 7 post exposure when C. neoformans was first isolated from the lungs. It was evident, therefore, that the presence of a stimulus was required to induce activation of host cellular responses. The organism was contained in the lungs until day 14 post infection with low rate of multiplication. This observation correlates with the activity of alveolar macrophages at days 7 and 14 in our study when these cells showed enhanced phagocytic activity and fungistatic effect on C. neoformans. When activation of alveolar macrophages dissipated at day 21 post infection, the organism was able to disseminate to the liver and it was contained in this organ, with very low rate of multiplication until day 28 post infection. At these two periods acquired immunity was also at its peak. Infected animals showed high level of protection against the lethal challenge, and survival time of these animals was of longer duration. These observations on the status of induced acquired immunity, and the restriction on the proliferation and dissemination of C. neoformans correlate with the enhanced phagocytic activity of peritoneal macrophages which exhibited a fungicidal effect on the organism. At day 42 post infection, however, the organism was able to proliferate rapidly in many animals, and disseminated to all organs including the central nervous system.

Phagocytosis of C. neoformans by human cells has been known to occur in vitro (6, 10). Diamond, 1972, (13) has shown that neutrophils and monocytes from patients with cryptococcosis had no defect in intracellular killing of C. neoformans when compared with leukocytes from

normal volunteers. Gentry and Remington (20) have observed that monolayers of mouse peritoneal macrophages infected in vitro with C. neoformans, resisted destruction by the organism. These macrophages were isolated from mice infected with obligate intracellular protozoa. However, the intracellular fate of C. neoformans within peritoneal macrophages was not studied. Mitchell and Friedman have noted killing of C. neoformans by rat peritoneal macrophages (40), but intracellular killing was very low and most yeast cells regardless of strain or capsular size, remained viable after ingestion.

The results of this investigation demonstrated that alveolar and peritoneal macrophages play a significant role in cellular immune response in certain stages of cryptococcal infection. This role was expressed in retarding the progression of infection, limiting multiplication and dissemination of C. neoformans to different organs. Also by exhibiting fungistatic effect of alveolar macrophages at day 14 and fungicidal effect by peritoneal macrophages at days 21 and 28, on the organism. These effects, however, were transient and both macrophage populations provided a favorable environment for the intracellular growth of C. neoformans on days 42, 56 and 84 post infection. Diamond and Bennett observed that C. neoformans was able to grow within human macrophages (15). They found that the organism was actively ingested by macrophages cultured from human peripheral blood monocytes; but these macrophages did not acquire the ability to kill or inhibit intracellular growth of C. neoformans. Also, they noted that there were no detectable differences between macrophages from normal subjects and

those from patients with cryptococcosis. The system for obtaining macrophage populations and the methodology they used were different from those used in this investigation where all alveolar and peritoneal macrophages were isolated from mice at different stages of cryptococcosis. Nevertheless, there was supportive evidence presented that these cells were not able to kill C. neoformans or inhibit its intracellular multiplication at certain stages of progressive cryptococcosis which was evident on days 42, 56 and 84 post infection. This observation raises the possibility that macrophages may have a non-protective role during progressive cryptococcal infection, although they played a significant role in protecting mice on days 21 and 28 post infection. Therefore, rather being the main effector arm of acquired immunity against progressive cryptococcosis; the macrophages may contribute to the pathogenesis of C. neoformans. This was expressed on days 42, 56, and 84 post infection, by the dissemination of the organism to the central nervous system, its rapid proliferation to all internal organs and the increased rate of mortalities among infected animals challenged at these periods. Louria and Brayton (32), observed that germination of Candida yeast cells occurred within phagocytes and filaments grew from the cells. Stanley and Hurly (62) noted that Candida was capable of intracellular growth within mouse peritoneal macrophages and proposed that such phagocytic cells might promote progressive candidiasis by acting as vectors for the multiplying Candida.

At day 56 post infection, the isolation of C. neoformans from lungs and brains of 33.33% of animals sacrificed at this period, while

spleens and livers of these animals were culturally negative to C. neoformans; suggests that there is another line of dissemination besides the hematogeneous spread which was postulated by Wilson (64). Ritter and Larsh (54) observed that C. neoformans was recovered from all organs of mice infected by intranasal instillation of the organism at the fourth and sixth weeks post infection. However, at the eighth week the lungs appeared as a sole site of infection and after 12 weeks post infection 5% of infected mice had the organism only in the lungs and brains. In the present study, enhanced phagocytic activity of peritoneal macrophages to C. neoformans occurred at day 56 post infection. This enhancement, however, was accompanied with intracellular multiplication of the organism which at 48 hrs post in vitro infection of monolayers caused necrosis and disintegration of macrophages. These observations suggest that peritoneal macrophages might have crossed the blood-brain barrier and acted as mediator for the dissemination of C. neoformans to the central nervous system.

It has been postulated that activation of macrophages results from the interaction of macrophages with immunologically committed lymphocytes which undergo blastogenesis as a result of their contact with specific antigen (35, 46, 47). Therefore, it is possible that C. neoformans was able to stimulate lymphocytes at certain stages of infection to produce the soluble mediators (lymphokines) which ultimately may have caused the activation of macrophages at these stages of cryptococcal infection. The results showed that peritoneal macrophages exhibited a transient fungicidal effect on C. neoformans

at 21 and 28 days post infection, while alveolar macrophages exhibited a transient fungistatic effect on the organism at day 14 post infection. Both macrophage populations isolated at these periods were resistant to destruction by C. neoformans upon in vitro infection of their monolayers. As the cryptococcal infection progressed, however, a state of immunosuppression might have been produced as a result of rendering the lymphocytes unresponsive to C. neoformans. This immunosuppressive state was expressed in the following events: (a) complete impairment of phagocytic activity by alveolar macrophages at days 56 and 84 post infection, and by immune alveolar macrophages isolated 3 weeks after the lethal challenge, (b) dissipation of macrophage activation and consequently the conversion of peritoneal and alveolar macrophages on days 42, 56, and 84 to favor intracellular growth of C. neoformans and ultimately the susceptibility for destruction by the fungus, (c) the increased rate of mortality among infected mice challenged on day 42, 56 and 84 post infection; and the shorter survival time of these animals, (d) the rapid proliferation of C. neoformans at day 42 and consequently its dissemination to the central nervous system.

Many experimental studies have suggested that cryptococcal capsular polysaccharide could induce an immunological paralysis in the infected host. Goodman (21), has found that cryptococcal polysaccharide is present in the serum and/or cerebrospinal fluid of many patients with cryptococcal meningitis. Murphy and Cozad (44), by employing the hemolytic plaque technique noted that high concentration

of cryptococcal capsular polysaccharide induced immunological unresponsiveness possibly by neutrilization of anticryptococcal antibody and inhibition of antibody synthesis. Bulmer and Sans (7), have observed that soluble cryptococcal polysaccharide specifically inhibits phagocytosis of C. neoformans by human leukocytes and that phagocytosis of the organism in patients with cryptococcal meningitis was depressed. These investigators suggested that cryptococcal polysaccharide may play a role in the pathogenesis of cryptococcosis. Diamond, Root, and Bennett (13) demonstrated that inhibition of phagocytosis of C. neoformans occurred only at extremely high concentrations of polysaccharide rarely seen in serum or spinal fluid of cryptococcal patients. They postulated that the size of cryptococcal capsule was a very important factor in affecting the ingestion of C. neoformans by neutrophils, whereas other factors such as free capsular polysaccharide and specific antibody to C. neoformans, may have less important influences on the phagocytic activity of neutrophils in human cryptococcosis. Whether the state of immunosuppression which was induced in mice during progressive stages of cryptococcosis, was a result of production and accumulation of cryptococcal polysaccharide during these stages, was not determined in our investigation.

In antimicrobial cellular immunity, macrophage activation and increased resistance appear to result from the stimuli provided by specific immunological events in combination with non-specific stimuli (50). Nevertheless, the capacity of different macrophages to suppress ingested microbes is determined by the degree of the cell activation

at the time of encounter with the microbe, i.e. the higher levels of enzymes in activated, as compared with non-activated, macrophages (23, 43). The results in this study indicate that nonspecific activation of alveolar macrophages isolated from immune mice was manifested by the enhanced phagocytic activity to Histoplasma capsulatum but not to Cryptococcus neoformans. This nonspecific activation was not accompanied by intracellular killing of H. capsulatum neither by immune alveolar macrophages or immune peritoneal macrophages. These observations suggest that C. neoformans might have produced a specific immunosuppression on the phagocytic activity of immune alveolar macrophages but not on the peritoneal macrophages. The latter exhibited a fungicidal effect and a similar pattern of phagocytic activity on C. neoformans as did the peritoneal macrophages isolated from infected mice on day 21 post infection. Intracellular growth of H. capsulatum within immune alveolar and peritoneal macrophages and consequently their necrosis and destruction may be related to the absence of specific cryptococcal antigen in the incubation medium of these cells. On the other hand, it may be due to non-specific manifestation of the immunosuppression which was induced by C. neoformans on macrophage population and could have been extended to affect the behavior of H. capsulatum within the phagocytic cells.

Since alveolar and peritoneal macrophages exhibited alternations in their functional activity as was demonstrated by their behavior in vitro and the correlation of this behavior to their role in vivo; it would be beneficial to continue investigations along the

following lines:

1. To study the immunological function of both T-lymphocytes and B-lymphocytes at different stages of cryptococcosis and to correlate this function with the behavior of alveolar and peritoneal macrophages *in vitro* and their protective and non-protective roles *in vivo*.
2. To determine the level of cryptococcal capsular polysaccharide at different stages of cryptococcal infection and to correlate this level with the activity of macrophages and lymphocytes *in vitro* and *in vivo*.
3. To perform a comparative study on the enzymatic contents of alveolar and peritoneal macrophages isolated from infected mice at different stages of cryptococcosis and correlate the level of these enzymes to the functional activity of macrophages *in vitro* and *in vivo*.
4. To perform auto-radiographic study on macrophages isolated at certain stages of progressive cryptococcosis, when the state of immunosuppression has been induced. This study may involve: infecting macrophage monolayers *in vitro* with labelled *C. neoformans*, allowing different times for phagocytosis, washing extracellular cryptococci, detaching the macrophages from monolayers, injecting the parasitized macrophage suspension in normal animals intraperitoneally, and finally tracing their distribution to observe if they are able of crossing the blood-brain barrier and establishing infection of the central nervous system.

CHAPTER V

SUMMARY AND CONCLUSIONS

Female inbred Balb/cj mice were infected with 1.6×10^4 Cryptococcus neoformans via the airborne route. Groups of mice were autopsied or challenged intravenously with 1.7×10^6 viable C. neoformans at days 1, 3, 7, 14, 21, 28, 42, 56, and 84 post infection for pathogenesis and mortality studies. Alveolar and peritoneal macrophages were isolated from infected and noninfected mice at the same periods of autopsy, and used in the in vitro phagocytic studies.

The results indicate that the pathway of dissemination of cryptococcosis is from the primary pulmonary infection to the liver, spleen, and finally to the brain. Pulmonary cryptococcosis, however, was not established until day 7 post respiratory exposure to the organism. This observation raises the possibility that Cryptococcus neoformans may initially colonize certain foci in the upper respiratory tract such as mucous membranes or lymphoid tissues, prior to further multiplication and dissemination which leads to the establishment of a primary focus of infection in the alveoli of the lungs.

Subclinical respiratory infection with 1.6×10^4 C. neoformans conferred immunity on mice against a lethal challenge with 1.7×10^6

viable C. neoformans. This acquired immunity was detected at day 7, peaked at day 21 and 28, then it started to subside on day 42 post infection, at which time an increase rate of mortality occurred and continued to increase until day 56 when it was maintained at the same level until day 84 post infection.

Alveolar macrophages exhibited a fungistatic effect on C. neoformans at day 14, whereas peritoneal macrophages exhibited fungicidal effect on the organism at days 21 and 28 post infection. In vitro functional activities of alveolar and peritoneal macrophages were expressed in vivo by retarding progression of infection, inhibiting multiplication and proliferation of C. neoformans, and finally extending the survival time of immunized-challenged animals at these periods. However, this protective role of both macrophage populations was a transient one, and both cells provided a favorable environment for the intracellular growth of C. neoformans as a result of an immunosuppressive state which was induced in the host in more progressive stages of infection. Therefore, rather being the main effector arm of acquired immunity against progressive cryptococcosis, the macrophages may contribute to the pathogenesis of C. neoformans by acting as a mediator for the dissemination of the organism to the central nervous system.

Alveolar and peritoneal macrophages isolated from immune mice were able to phagocytize Histoplasma capsulatum, but they failed to resist destruction by the organism 48 hrs post in vitro infection of the monolayers.

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