FURTHER STUDIES ON IMMUNITY IN EXPERIMENTAL CRYPTOCOCCOSIS*

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PLATES 20 TO 23

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We have demonstrated previously (1) that mice infected with small numbers of highly virulent or large numbers of less virulent *Cryptococcus neoformans* resist challenge initiated 14 days later with ordinarily lethal inocula of this pathogen, but not with *Staphylococcus aureus*, *Klebsiella pneumoniae*, or *Mycobacterium tuberculosis*. Equal protection was achieved by injection of bacterial endotoxins 1 week prior to challenge. However, animals specifically immunized with living cryptococci were able to limit multiplication during the 1st week of infection, whereas those non-specifically immunized with endotoxin were not, although survival rates over a 3 month period were similar in both groups. It was also shown that when small numbers of virulent cryptococci were injected intravenously, tissue populations rose in all organs studied, including the brain, over the initial 7 days of infection. In some animals, progressive disease ensued leading to death with hydrocephalus, but the majority survived, and in these, fungal census fell gradually, so that at the end of 6 months, tissues were almost uniformly sterile.

Additional data gathered on the mechanisms of immunity in specifically and non-specifically protected mice challenged with *C. neoformans* form the basis for the present report. Four aspects of experimental immunity have been studied:

1. The ability of mice surviving infection with small numbers of cryptococci to resist ordinarily lethal challenge 3 to 4 months later, when tissue cryptococcal census had fallen to virtually zero

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2. Tissue populations in control and immunized animals 2 weeks after infection with large inocula

3. The possible correlation between protection and the appearance of antibody in the plasma of immunized animals

4. Histologic changes in the tissues of immune and control mice

Materials and Methods

Mice.—Male Swiss albino mice from Carworth Farms, New York (strain CFW) were used in all experiments. The animals weighed 16 to 19 gm at the start of immunization and 23 to 27 gm at the time of challenge. They were housed in metal cages and were given mouse pellets and water *ad libitum*.

Cryptococcus Strains.—These have been described previously (1). Strain 1148, a large capsule strain, produced death in 80 to 100 per cent of normal mice within 30 days after intravenous injection of 2.5×10^6 cells. It was subcultured on Sabouraud's glucose agar slants weekly and virulence was maintained by intermittent mouse passage. Strain B-27, a small capsule strain of *C. neoformans*, which rarely produced death after intraperitoneal inoculation of 5×10^7 cells, was also maintained by weekly subculture on Sabouraud's glucose agar.

Endotoxin.—Lots AE 1298 S_4 and AE 1688 S_4 of purified *Salmonella* endotoxin (pyrexal®) were employed in these experiments.

Immunization Schedule.—Specific immunity was achieved by 3 intraperitoneal injections of 2×10^7 cells of strain B-27 at 10-day intervals, the last being administered 2 weeks prior to challenge. Non-specific immunity was obtained by a single intraperitoneal injection of 100 to 200 μg of endotoxin 7 days prior to challenge.

In additional studies, cells were harvested from a 24 hour Sabouraud's glucose agar slant incubated at 30°C, suspended in physiologic saline, counted directly in a Neubauer-Levy hemocytometer chamber, and diluted to 2×10^3 cells per ml. 0.5 ml of the resulting suspension was injected into the lateral tail vein of each mouse. The number of cryptococci in the inoculum was checked by standard pour plate enumeration techniques.

Challenge Schedules.—Control animals and those immunized with strain B-27, endotoxin, or strain 1148, were always challenged intravenously with 2×10^6 cells of strain 1148, harvested from a 24 hour Sabouraud's agar slant culture and injected in a 0.50 ml volume into a lateral tail vein. Groups of animals were sacrificed at appropriate intervals for histologic study, determination of circulating antibody, and analysis of tissue populations. Others were observed for mortality rates over a period of 3 months. Mice immunized intraperitoneally with living cryptococci were challenged 14 days after the last immunizing injection. Those given endotoxin were infected 7 days later, and animals infected with 10^3 cells of strain 1148 intravenously were challenged 1 week, 1 month, 3 months, or 4 months later.

Tissue Population Studies.—Animals were killed by etherization. Lungs, brain, both kidneys, and the combined liver and spleen were removed aseptically and were emulsified separately in 2 ml of distilled water with a teflon tissue homogenizer. Sabouraud's agar pour plates were made of serial 100-fold dilutions of the tissue homogenate. Plates were incubated at 30°C and colony counts were determined at 2, 4, and 7 days.

Antibody Determination.—The method used was similar to that described by Neill, Castillo, Smith, and Kapros (2). Specimens were obtained from normal and immunized mice 2, 4, 7, and 14 days after challenge. Groups of 5 mice were lightly anesthetized and were exsanguinated through the axillary artery. Heparinized blood specimens from the 5 animals were pooled and the plasma removed and stored at −20°C until used. In each test, 2-fold saline dilutions of each specimen were made. Cryptococcal strains 1148 or B-27 were harvested in saline from a

24 hour Sabouraud's glucose agar slant, killed by heating at 60°C for 1 hour, and diluted to a concentration of 2×10^7 cells per ml. 0.50 ml of the suspension was added to each plasma dilution so that the final volume of the test was 1 ml. The lowest plasma dilution used was 1:4. Each specimen was studied with both cryptococcal strains. All tubes were shaken vigorously manually, incubated at 37°C for 2 hours, and then stored at 4°C. Tubes were read independently by two observers for macroscopic agglutination 1, 2, 4, and 6 days after refrigeration. The growth of strain 1148 in control and immunized plasma was also studied at each time interval. An inoculum of 10^3 cryptococcal cells suspended in 0.5 ml of tryptose phosphate broth was added to 0.5 ml of each plasma dilution. Tubes were incubated at 37°C, and after 24 hours, growth was determined by Sabouraud's agar pour plate enumeration techniques.

Histologic Studies.—Normal and immunized mice were sacrificed 2, 4, 7, and 14 days after challenge. Tissues were placed in formalin and sections of the liver, spleen, kidneys, lungs, and brain were stained with hematoxylin and eosin or by the periodic acid–Schiff method.

Statistical Analysis.—The effect of immunization was studied by analyzing cumulative mortality curves and 3-month survival in control and immunized groups. The time of deaths following challenge was plotted on logarithmic probability paper. The median time of death and significance of protection were calculated according to the method of Litchfield and Wilcoxon (3). Standard T tests were used in analyzing tissue populations. The significance of survival in immunized animals and controls was compared by the method of chi square.

EXPERIMENTAL

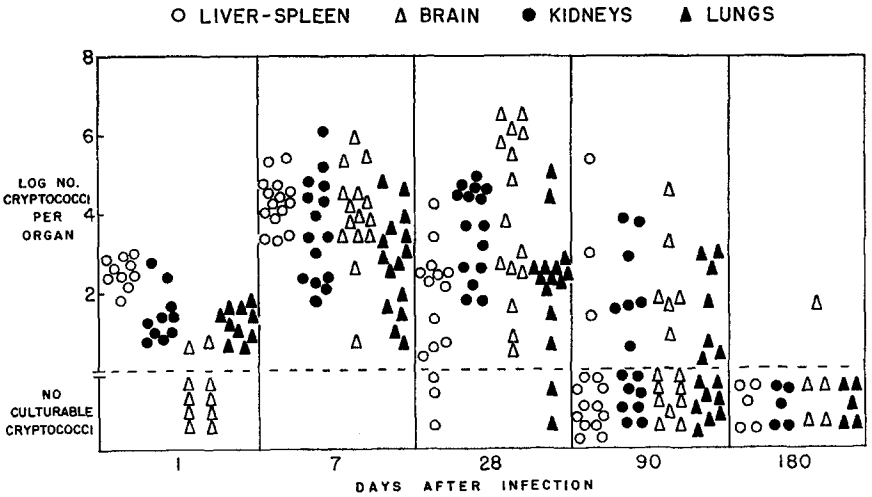
1. Immunity Achieved by Infection with Small Numbers of Virulent Cryptococci.—

Five separate experiments were performed in which a total of 265 mice were infected with 10^3 cells of strain 1148 intravenously. Fifty of the animals were used 1 week after infection either for tissue population studies or for challenge with an ordinarily lethal inoculum. Of the remaining 215 mice, 80 (27 per cent) died of the infection within 3 months, the number of deaths in individual experiments ranging from 10 to 50 per cent. Tissue cryptococcal census was determined in 35 survivors 1 to 6 months after inoculation, and an additional 93 animals were challenged intravenously with 2.5×10^6 cells of virulent strain 1148.

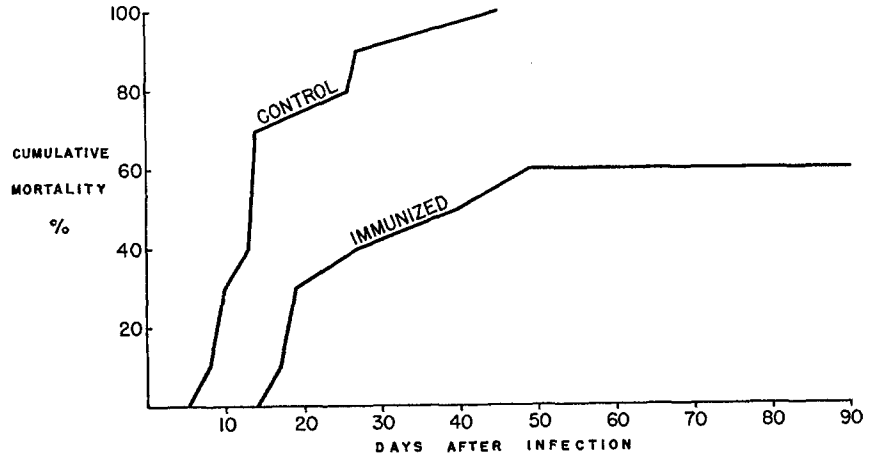
Three challenge experiments were performed at 1 week after infection with 10^3 cells, 3 at 1 month, 3 at 3 months, and 1 at 4 months. On each occasion, 10 control mice of the same age and approximately the same weight were challenged simultaneously.

The results of tissue population studies are depicted in Text-fig. 1. Cryptococcal census rose in all tissues studied during the initial 7 to 28 days, increase being maximum in the brain. In the 15 animals observed 3 months, and the 5 studied 6 months after infection, fungus populations fell markedly. In 12 of the 20 animals, all tissues studied were either sterile or contained less then 2.5×10^1 cryptococci and in 7 of the remaining 8 animals, only a single tissue had a fungal census of greater than 10^2, large numbers of cryptococci being recovered from the 20th animal.

No protection was observed in any of the 3 experiments in which mice were challenged with a lethal inoculum 1 week after infection with 10^3 cells intravenously. Similarly, no protection was observed in 1 of the 3 challenge experiments performed 1 month after inoculation with 10^3 cells, but in the other



TEXT-FIG. 1. Cryptococcal populations in mouse tissues following intravenous infection with 10^8 cells of *C. neoformans*, strain 1148.

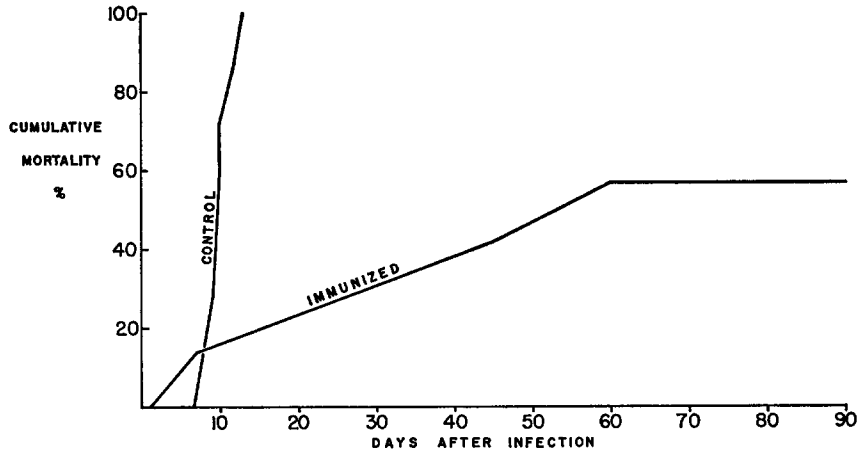


TEXT-FIG. 2. Mortality in mice infected intravenously with 2.5×10^6 cells of *C. neoformans*, strain 1148, 1 month after intravenous infection with 10^8 cells of the same strain.

2 experiments, death was delayed and 3 month survival significantly increased ($P < 0.01$); all 18 control mice died within the study period, whereas 9 of 18 (50 per cent) of the immunized animals survived. One of the 2 experiments in which protection was observed is shown in Text-fig. 2. Delay in death and increased survival were observed in all 5 experiments performed at the 3 to 4 month interval; 30 of 65 (42.2 per cent) of immunized animals survived the

3 month challenge period, whereas all 47 controls died. One of these studies is depicted in Text-fig. 3 and all 11 challenge experiments are summarized in Table I.

2. Two-Week Tissue Population Studies.—Because mice previously exposed to cryptococci showed limited multiplication of the fungus during the first 7 days after challenge, and those immunized with endotoxin did not (1), it seemed important to determine whether endotoxin-protected animals survived despite high tissue populations or whether they gained control of the infection between the 7th and 14th day, a period during which control animals began to die with hydrocephalus. In 2 experiments, groups of 5 mice were



TEXT-FIG. 3. Mortality in mice infected intravenously with 2.5×10^6 cells of *C. neoformans*, strain 1148, 3 months after intravenous infection with 10^3 cells of the same strain.

sacrificed for determination of brain fungus census 1, 7, and 14 days after infection. These studies are summarized in Text-fig. 4. Progressive multiplication was observed in control animals over the 14 day period. In mice immunized with strain B-27, cryptococcal multiplication was considerably reduced, populations averaging 1 log unit less than those found in controls at both the 1 and 2 week intervals. These differences are statistically highly significant ($P < 0.01$). In endotoxin-treated animals, brain populations at the end of 7 days were indistinguishable from those of the controls, but 1 week later, (14 days after infection), brain census was similar to that observed in specifically immunized mice and differed from controls by more than 1 log unit, a difference with high statistical significance ($P < 0.01$). Thus, the endotoxin-immunized animals appeared to gain control of the infection between the 1st and 2nd week after challenge.

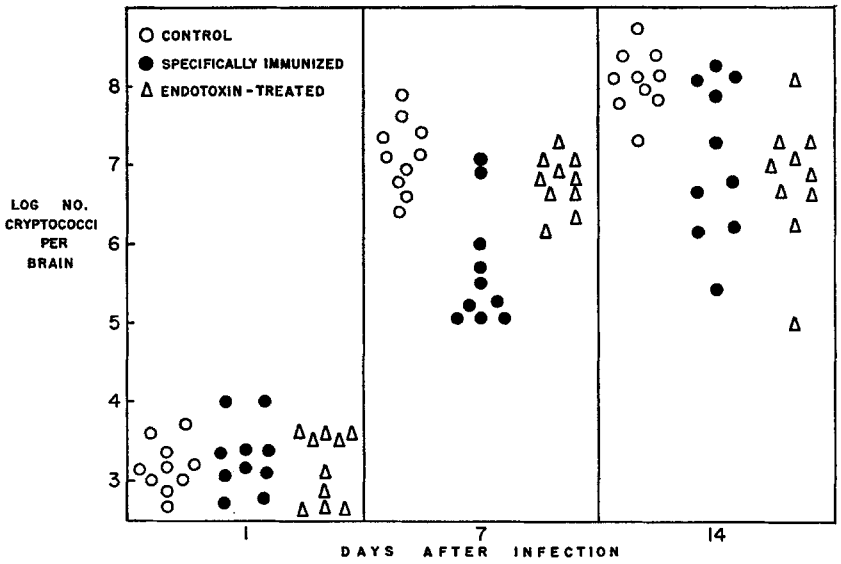
3. Antibody and Plasma Inhibition Studies.—Since endotoxin-treated animals showed no ability to limit the infection during the 1st week, but gained control thereafter, it seemed possible that this phenomenon might be associated with the appearance of circulating antibody. Plasma was studied from the same animals whose tissue populations are depicted in Text-fig. 4. The results are shown in Table II. No agglutinating antibody was demonstrable in the plasma

TABLE I

Mortality in Mice Infected Intravenously with 2.5 × 10^6 Cells of C. neoformans Strain 1148 at Various Times after Intravenous Infection with 10^3 Cells of the Same Strain

Time of challenge	Experiment No.	LD_{50}		3 mo. Survivors	
		Control	Immunized	Control	Immunized
		days	*days*		
1 wk.	1	20	22	1/10	0/10
	2	16	17	0/10	0/10
	3	5	5	0/9	0/9
1 mo.	4	50	55	3/10	3/10
	5	5	>90	0/8	5/8
	6	14	40	0/10	4/10
3 mos.	7	20	71	0/10	5/10
	8	4	>90	0/10	7/10
	9	9	53	0/10	4/10
	10	18	80	0/10	11/28
4 mos.	11	10	62	0/7	3/7
Survivors, experiments 5 to 11.....				0/65	39/83 (47 per cent)

of normal mice at 2, 4, 7, or 14 days using either strain 1148 or B-27 as the antigen. Agglutinating antibody titers of 1:4 to 1:8 were found in 1 of the 2 plasma specimens of specifically immunized mice studied 4 days and in 1 of the 2 specimens obtained 2 weeks after challenge. Both the 2 day and 7 day specimens showed no agglutination on repeated testing with both strains of *C. neoformans*. Thus, between the 4th and 14th day, 2 of 6 specimens studied showed low agglutination titers. The findings in the endotoxin-treated animals were in striking contrast to those in the normal and specifically immunized. No agglutination was observed in the 2 and 4 day specimens, but in the 1 week plasmas, titers varied from 1:8 to 1:16 with strain B-27 as the antigen, and from 1:16 to 1:32 with homologous strain 1148 as the antigen. At the 2 week interval, titers varied from 1:4 to 1:8 with both antigens.



TEXT-FIG. 4. Brain cryptococcal populations in specifically and non-specifically (endotoxin) immunized mice 7 and 14 days after intravenous challenge with 2.5 × 10^6 cells of *C. neoformans*, strain 1148.

TABLE II

Agglutinating Antibody Titers in Plasma of Mice Immunized Specifically and Non-Specifically (Endotoxin) against C. neoformans

Days after challenge with strain 1148.....	2	4	7	14
Exp. No.				
1 Specifically im-	<1:4	<1:4	<1:4	<1:4
2 munized*		1:4 to 1:8		1:4 to 1:8
1 Endotoxin-treated			1:8 to 1:32	1:4 to 1:8
2	<1:4	<1:4	1:8 to 1:32	1:4 to 1:8
1 Control	<1:4	<1:4	<1:4	<1:4
2				

* Results with antigens B-27 and 1148 were similar and are pooled.

When 10^3 cryptococci were added to 1:4 or 1:8 dilutions of normal mouse plasma, populations increased 1000-fold in 24 hours. Plasmas obtained from specifically immunized and endotoxin-immunized animals 2 to 14 days after challenge showed no evidence of ability to limit cryptococcal growth.

4. Histopathology of Cryptococcus Infection.—

Central nervous system infection with *C. neoformans* was characterized by numerous focal lesions scattered diffusely throughout the brain (Fig. 1). Organisms were usually but not invariably present in these areas. Although cryptococci could be demonstrated by the usual hematoxylin and eosin stain, they were more consistently visualized by the periodic acid–Schiff stain. A remarkable feature of these lesions in control animals (including those sacrificed at 2 weeks) was the paucity of the inflammatory reaction; neither cellular infiltration, glial response, nor neuronal change could be found, even within and surrounding large or confluent areas of cryptococcal invasion. In both immunized groups, the lesions were equally bland in specimens obtained 2 days and 4 days after infection. At 1 week, however, a definite and occasionally very prominent inflammatory response could be detected, the exudate consisting of a mixture of neutrophils and mononuclear cells (Fig. 2). Cellular response in specifically immunized and in endotoxin-immunized mice showed no differences.

The meninges were also involved by infiltration of acute and chronic inflammatory cells into the leptomeninges. The inflammatory reaction began early and was progressive. The treated and control groups did not differ (Fig. 3).

A focal vascular inflammatory response was elicited at the 1 and 2 week intervals in both control and immunized groups, but was more frequent and more severe in the latter (Fig. 4). The lesions were thought to be focal endothelial proliferations, but infiltration by mononuclear cells could not be ruled out. Infrequently, neutrophils were present, but again it was sometimes difficult to decide whether these were within the lumen or the wall of the vessel.

Microscopic sections of heart, lung, liver, spleen, and kidneys were also examined. Organisms were not seen in the spleen, but infected animals showed reactive hyperplasia of the lymphoid follicles. In the other organs, cryptococci could be found and frequently elicited a focal inflammatory reaction consisting of a mixture of polymorphonuclear leukocytes, lymphocytes, and histiocytes. Unlike the situation in the central nervous system, no difference in the extent of the lesions or in the cellular responses could be detected at any of the time intervals studied. Lesions containing the organism but with little inflammatory response were occasionally found in control and treated groups infected for 2 weeks, as were hepatic granulomas devoid of organisms.

DISCUSSION

It has long been held that host cellular and antibody defense mechanisms are inadequate in cryptococcosis. This has been based on the histologic observations of myriads of cryptococci in tissues of both animals and man in the absence of an inflammatory response, and on past failures to demonstrate immunity in experimental animals or circulating antibody in the serum of man during the course of cryptococcal infection. Studies from several laboratories have indicated that this thesis is no longer tenable. We have demonstrated previously that infection with small numbers of highly virulent cryptococci or large numbers of less virulent strains intraperitoneally protects animals against intravenous challenge 2 weeks later (1). In the experiments reported herein, we have shown that death is delayed and survival markedly increased if mice are challenged 1 to 4 months after infection with 10^3 cells of a virulent strain intravenously, protection being consistently observed even when tissue cryptococcal census is extremely small. In addition, Gadebusch (4) successfully immunized mice with purified cryptococcal capsular polysaccharide plus adjuvant. Abrahams and Gilleran (5) found increased resistance following vaccination with suspensions of

formalin-killed cells. In their studies, in contrast to the prolonged immunity noted in our experiments, the induced protection was short-lived and resistance was significantly decreased when challenge was made 21 instead of 14 days after immunization. Vogel, Sellers, and Woodward (6), using fluorescent techniques, have demonstrated antibody in the serum of patients with cryptococcosis, as have Pollock and Ward (7) utilizing a hemagglutination test, and Bennett, Tynes, and Hasenclever employing complement-fixation techniques (8). It would appear, then, that antibodies are formed against invading cryptococci, and that at least under certain experimental circumstances, immune mechanisms may be quite effective in limiting progressive multiplication of the fungus.

Gadebusch, and Abrahams and Gilleran, demonstrated diminished protection when large amounts of vaccine or purified capsular polysaccharide were used, thus supporting the contention of Salvin (9) that the failure to demonstrate a brisk antibody response may be related to paralysis of immune mechanisms induced by antigen excess.

The current experiments and those previously reported from this laboratory suggest that this is probably of small clinical significance. Multiplication occurred in all tissues for a period of 1 week to 1 month after inoculation with small numbers of cryptococci but thereafter, the majority of animals gained control of the infection. Since the greatest amount of circulating polysaccharide antigens should be expected when tissue populations are maximum, it is difficult to explain the ability of the mouse to gain control of the infection at this time if one accepts the thesis that immune paralysis due to antigen excess plays any significant role in the course of cryptococcosis. More likely, past failure to demonstrate circulating antibodies has been due to the use of techniques which were not sensitive enough to detect them. Recent advances in methodology (6–8) may now permit demonstration of significant titers of circulating antibody in the majority of patients and experimental animals with cryptococcosis.

It is interesting that agglutinating antibody was present more consistently and in higher titer in endotoxin-pretreated animals than in those specifically immunized. Although administration of endotoxin is known to increase antibody response (10), it also modifies many other host defense mechanisms, and this may result in increased resistance to homologous or heterologous infections. These alterations include increased phagocytic capacity of the reticuloendothelial system (11), augmented leukocyte response (12), enhanced phagocytosis by polymorphonuclear leukocytes (13), release of preformed antibody (14), increase in plasma bactericidins (15), and enhanced ability to resist the lethal effects of microbial toxins (16). In the present studies, however, only the augmented ability to form antibody appears important. There was no change in the distribution of microorganisms or their initial growth over the first 7 days, and such change would have occurred if reticuloendothelial activity or plasma

fungicidal substances had been significantly altered. An increased polymorphonuclear leukocyte response was seen in histologic sections 7 days after infection, but there was no evidence of control of the infection in endotoxin-treated mice during this period. Conversely, specifically immunized mice, which showed an identical cellular response, did limit cryptococcal growth during the initial 7 days. Failure to demonstrate antibody 2 and 4 days after challenge indicates that the release of preformed antibody also played no role in these experiments. The significant difference in tissue census between controls and endotoxin-treated animals 2 weeks after challenge (when control animals began to die) suggests strongly that the major factor in increased survival was limitation of microbial multiplication rather than an ability to resist microbial toxins. Salvin has performed studies suggesting that cryptococci may contain endotoxin-like material (17), but there is no conclusive evidence that death in cryptococcosis is related either to an exotoxin or to cryptococcal endotoxins.

Although the importance of immune mechanisms in control of cryptococcosis appears clear, it is not known whether limitation of cryptococcal multiplication is related to circulating antibody, tissue-bound antibody, or alterations in host cellular defenses. As noted above, tissue cryptococcal populations could not be correlated with the enhanced cellular response observed in both specifically protected and endotoxin-treated animals. Gadebusch was able to delay death in mice infected with *C. neoformans* by treatment with immune rabbit serum (18). Since cryptococci and serum were injected intraperitoneally within 2 hours of each other, the resulting intraperitoneal agglutination may have reduced the size of the inoculum, a possibility which makes interpretation of the results difficult. In the present experiments, endotoxin-treated mice inhibited fungus growth only after circulating antibody could be readily demonstrated. However, specifically immunized animals were able to limit cryptococcal multiplication during the initial 7 days of infection despite low circulating antibody titers. Since control of the infection could not be closely related to either circulating antibody or the augmented cellular infiltration, it would seem reasonable to attribute inhibition of fungal growth to the presence of tissue-bound antibody.

Our findings do not derogate the importance of the initial sparse inflammatory response which may permit early dissemination to distant sites shortly after the establishment of a focal lesion. Indeed, it would appear to be a reasonable hypothesis that strongly encapsulated cryptococci both fail to elicit and also resist host cellular defenses until antibody is present, at which time the production of capsular material is reduced or capsular substances diffuse away from the cell wall, thus permitting phagocytosis by host defense cells. This concept is consistent with the studies of Gadebusch, who showed that large capsule strains of *C. neoformans* are not phagocytosed by polymorphonuclear leukocytes whereas small capsule strains are (18), and with the experiments of Iverson and Kase (19), who demonstrated that in older lesions, the fungus cells

appear to have less capsular material surrounding them and are phagocytosed by large mononuclear cells.

Since host immune mechanisms can be effective in cryptococcosis, it seems likely that this disease is far more common than has been heretofore recognized. It is indeed possible that in man, cryptococcosis, much like histoplasmosis, is usually a short-lived, benign illness limited to the lungs which only infrequently progresses to severe dissemination. The apparent dependence on immune mechanisms also lends further weight to the contention that the susceptibility of some patients with reticuloendothelial disease to torulosis is related to their well documented inability to respond adequately to certain antigens rather than to any change in the tissue biochemical *milieu* which might provide additional growth factors for invading cryptococci.

SUMMARY

Following infection with 10^3 cells of *Cryptococcus neoformans*, progressive multiplication occurred in all tissues for 7 to 28 days. Thereafter, most mice gained control of the infection so that 3 to 6 months after inoculation, tissues were usually sterile or contained only small numbers of cryptococci. Survivors challenged 1 to 4 months after infection with 10^3 cells lived longer than controls and had an increased survival rate. Pretreatment with bacterial endotoxin also resulted in protection against cryptococcal challenge. Endotoxin-protected animals showed no ability to limit the infection until circulating antibody could be demonstrated.

Histologic studies revealed an increased cellular infiltration in the brains of specifically protected and endotoxin-protected mice 1 to 2 weeks after challenge. However, control of cryptococcal multiplication *could* be correlated with the presence of antibody but *could not* be correlated with the enhanced cellular response.

Although protection against cryptococcal challenge appears to be antibody-dependent, it is unclear whether protective antibody is to be found in plasma or is tissue-bound.

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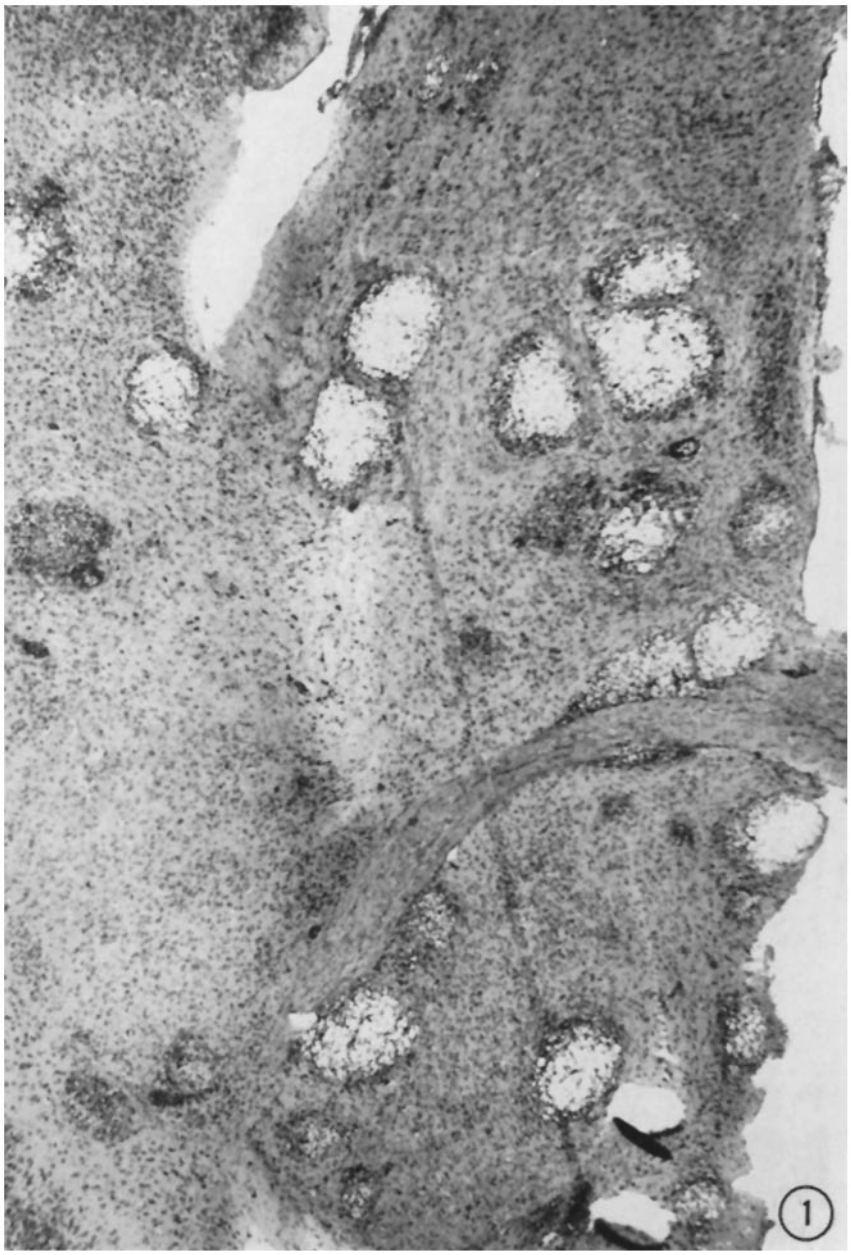
EXPLANATION OF PLATES

PLATE 20

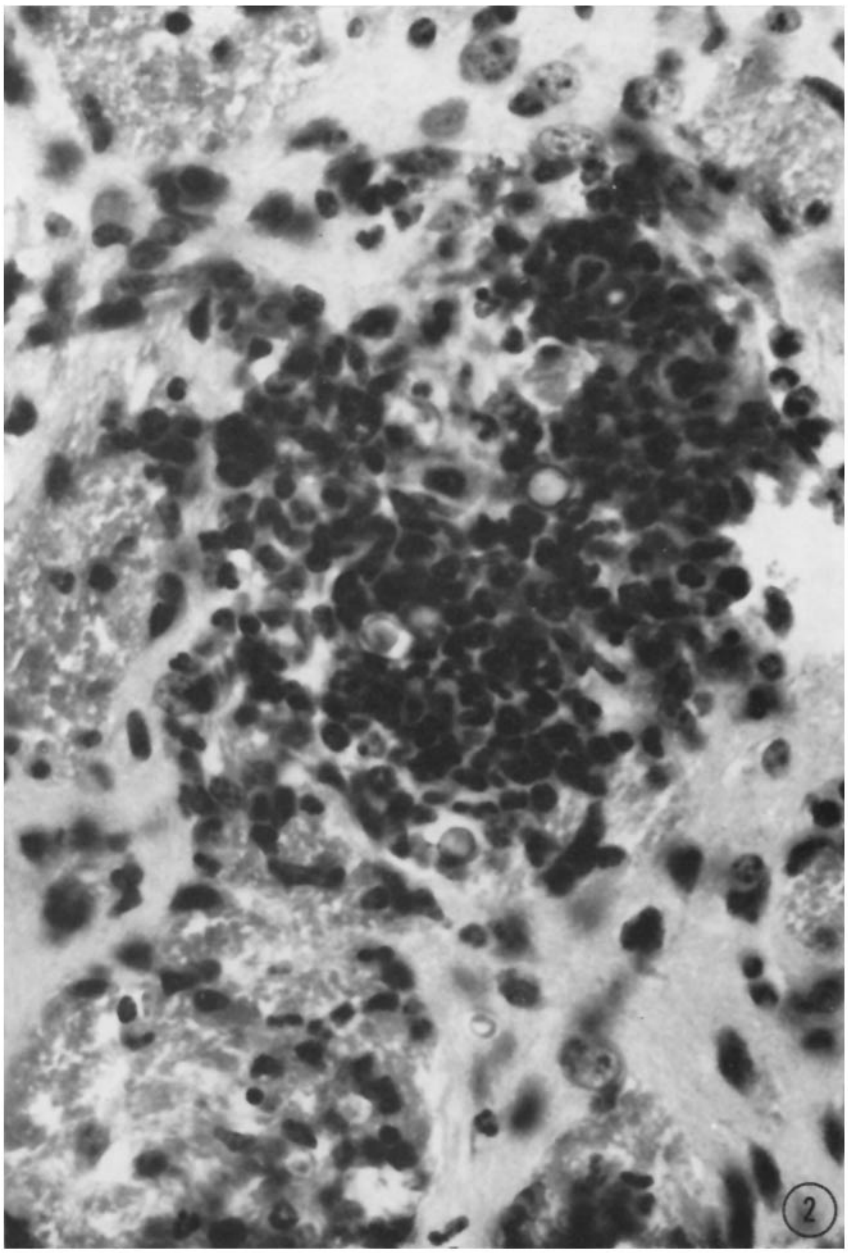
FIG. 1. Bland cyst-like lesion in brain of control animal 2 weeks after inoculation. Hematoxylin and eosin. × 150.



(Louria *et al.*: Immunity in cryptococcosis)

PLATE 21

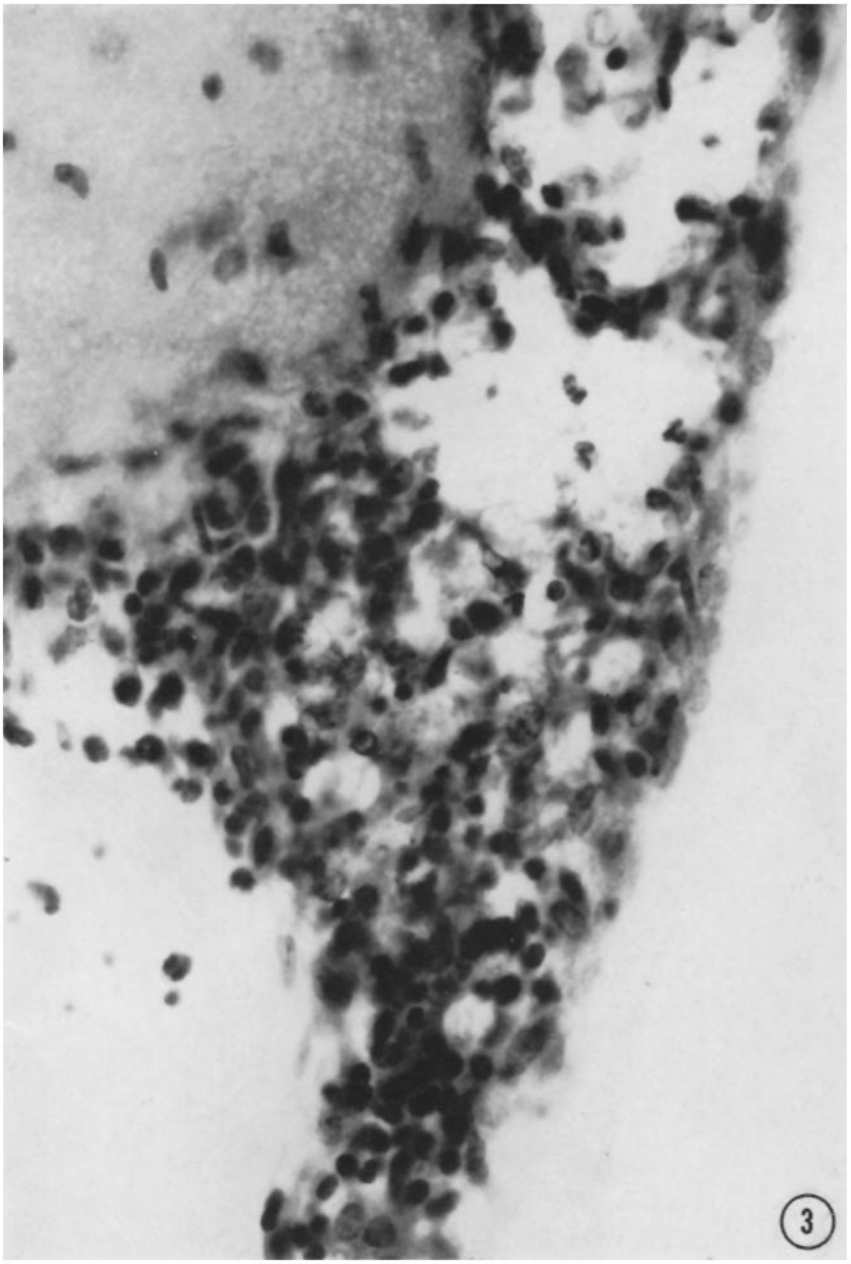
FIG. 2. Brain abscess containing inflammatory cells and cryptococcus organisms in immunized animal 2 weeks after inoculation. Hematoxylin and eosin. × 500.



(Louria *et al.*: Immunity in cryptococcosis)

PLATE 22

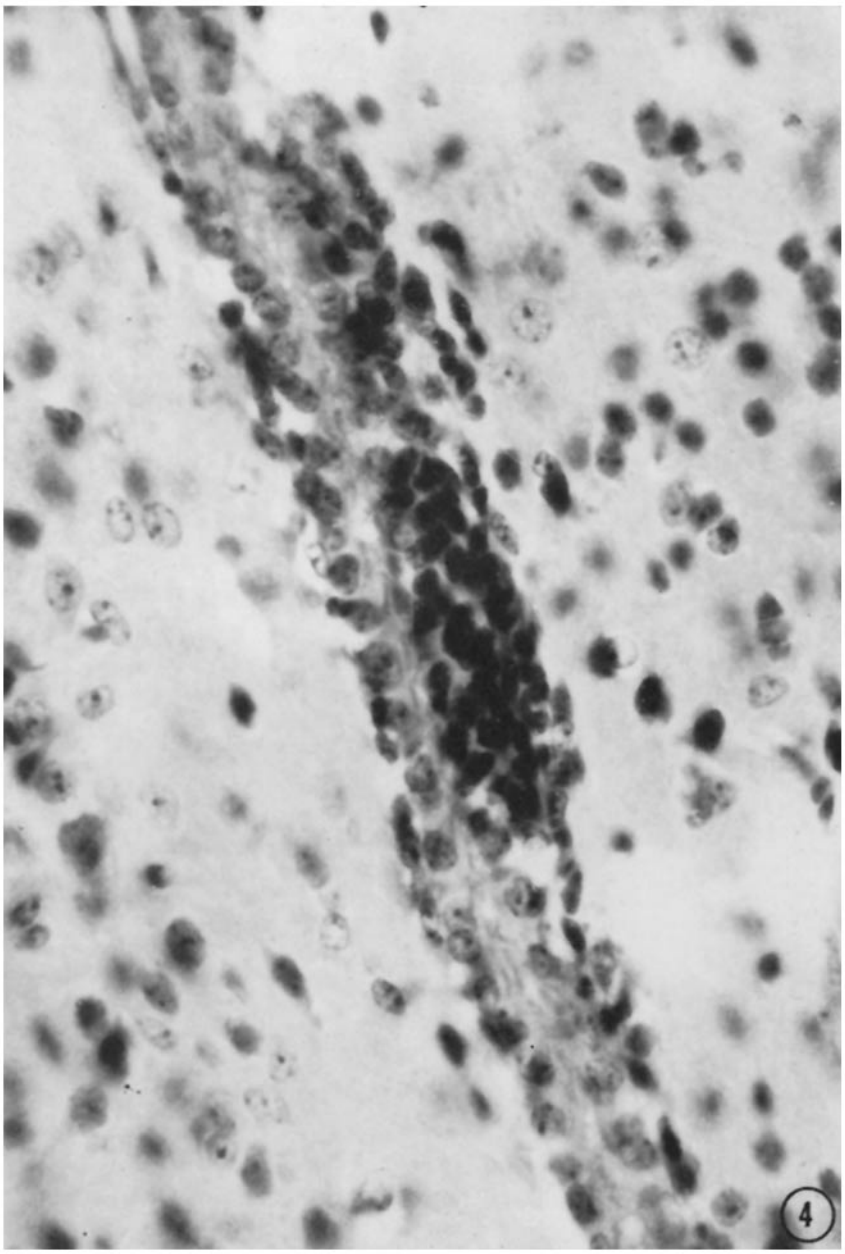
FIG. 3. Cryptococcal meningitis in immunized animal 1 week after inoculation. Hematoxylin and eosin. × 500.



(Louria *et al.*: Immunity in cryptococcosis)

PLATE 23

FIG. 4. Focal endothelial proliferation in small cerebral vessel of immunized animal 2 weeks after inoculation. Hematoxylin and eosin. × 500.



(Louria *et al*: Immunity in cryptococcosis)