SPECIFIC AND NON-SPECIFIC IMMUNITY IN EXPERIMENTAL CRYPTOCOCCOSIS IN MICE*

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Host defense mechanisms often appear to be inadequate following cryptococcal invasion (1–4). Significant antibody response has not been demonstrated with any regularity in man or experimental animals during torula infection. Furthermore, cellular response in cryptococcosis is frequently sparse. The apparent inadequacy of host antibody and cellular defenses during torula infection has been related to the presence of a large capsule, surrounding the cryptococcus cell, which consists primarily of several polysaccharides (5).

Benham (6) was able to produce antibodies to cryptococci in rabbits only after the capsule had been removed with hydrochloric acid. Evans and Kessel (7, 8) and Neill, Castillo, Smith, and Kapros (9), however, demonstrated precipitating and agglutinating antibodies in the rabbit following immunization with large numbers of heat- or formalin-killed cryptococci. These antibodies were clearly shown to be directed against the capsular material. The rabbit does not ordinarily develop progressive cryptococcosis so that no inference could be drawn as to the protective effect of such immunization in this animal.

In recent studies, Gadebusch (10) reported that he could delay death in mice following cryptococcal challenge by the administration of immune rabbit serum. These as yet unconfirmed studies suggested that immune rabbit serum not only contained antibody directed against the cryptococcal capsule, but also was able to modify the course of a lethal cryptococcal infection. However, the challenge organisms were suspended in mucin, and the antisera and challenge were both given intraperitoneally within 2 hours of each other. It is quite possible that the apparent protection was related to clumping of fungi by the agglutinating antisera, thus in actuality reducing the size of the inoculum. Furthermore, mucin itself can at first reduce and later increase resistance non-specifically to many infections (11). Thus temporary clumping of fungus cells by antisera might retard the acute infection long enough to allow non-specific resistance to develop.

Gadebusch (12) also reported that death was delayed in mice infected with a lethal

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inoculum of cryptococci by prior injection of purified capsular polysaccharide combined with a resin. The specificity of such protection was not investigated. No significant protection was observed when purified capsular polysaccharide was used alone as the immunizing agent.

It is thus unclear as to whether previous experience with cryptococci or cryptococcal products can modify the course of subsequent cryptococcus infections. In the present studies, mice were inoculated with small numbers of highly virulent or large numbers of less virulent living cryptococci. Death was delayed and survival rate was increased following subsequent challenge with ordinarily lethal inocula. The immunity thus induced appeared to be specific and did not confer protection against heterologous challenge. Non-specific immunity of similar degree could be produced by injection of bacterial endotoxin 1 week prior to challenge.

Experiments were also performed in which the course of infection produced by small numbers of cryptococci was studied in detail in normal non-immunized mice. Fungus tissue census rose markedly during the 1st week in all organs examined. Thereafter, tissue populations fell progressively with eventual eradication of fungi in most animals. This suggests that host defenses are in reality quite effective in mice if infection is initiated with non-overwhelming numbers of cryptococci.

Materials and Methods

Mice.—

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

Cryptococcus Strains.—

Three strains of *Cryptococcus neoformans* were utilized in these studies:

(*a*) Strain 1148, a large capsule strain, was isolated from the cerebrospinal fluid of a patient with chronic torulosis in 1954. The total cell size in mouse tissues varied from 12 to 20 μ approximately $\frac{2}{3}$ of which was capsule. A consistent virulence for mice was demonstrated when the strain was maintained by twice weekly passage on Sabouraud's agar at 30°C. Eighty to 100 per cent of mice died within a month following intravenous injection of 2.5×10^6 cells or intracerebral inoculation of 5×10^4 cells, most of the animals exhibiting gross hydrocephalus. Less frequent subculturing on agar was associated with a decrease in virulence. In later experiments, therefore, when virulence had diminished this strain was used for immunization, and will be referred to as avirulent strain 1148. In such studies mice were challenged with virulent strain 1149, which was obtained by mouse passage of strain 1148.

(*b*) Strain B-27, a small capsule strain, was isolated from a pulmonary toruloma in 1955.[1] The total cell size both *in vitro* and *in vivo* was 4 to 9 μ, the capsule comprising less than half the size of the entire cell. The strain grew moderately well at 37°C. It produced deaths in

[1] This strain was sent to Dr. C. W. Emmons at the National Institutes of Health by Dr. L. A. Weed. The author obtained the microorganism in turn from Dr. Emmons.

mice uncommonly following inoculation of 5×10^7 cells intraperitoneally, or 5×10^4 cells intracerebrally. When 2.5×10^6 cells were injected intravenously, the mortality rate was variable, 10 to 60 per cent dying within 3 months with hydrocephalus.

(*c*) Strain JN, a small capsule strain, was recovered from the cerebrospinal fluid of a patient with chronic torula meningitis in 1956. It was avirulent for mice and did not multiply significantly at 37°C. It could, therefore, be classified as *Cryptococcus neoformans* var. *innocuous*.

Immunization Schedules.—

Strains B-27 and JN were harvested in saline from Sabouraud's glucose agar slants after incubation at 30°C. for 48 (strain B-27) or 72 (strain JN) hours. The size of the inoculum was determined by direct hemocytometer counts in a Neubauer-Levy chamber. Twenty million cells were inoculated intraperitoneally in 0.5 ml. Three such injections were made at 10 day intervals, the last injection being 2 weeks prior to challenge.

Strains 1148 and 1149 were harvested in saline from Sabouraud's agar slants after 24 hours incubation at 30°C. Twenty-five thousand cells were inoculated intraperitoneally on a single occasion 14 days prior to challenge.

When strain 1148 had diminished in virulence and was used for immunization, 2.5×10^6 cells harvested from a 24 hour Sabouraud's agar slant were inoculated intravenously on a single occasion 2 to 8 weeks prior to challenge. Mice all appeared well at the time of challenge.

Virulent strain 1148 was also killed by heating at 60°C. for 30 minutes. Twenty million cells were inoculated intraperitoneally employing the same injection schedule described above for strains B-27 and JN.

Control mice in each experiment were given 0.5 ml. of physiologic saline intraperitoneally at 10 day intervals, the last injection being made 2 weeks prior to challenge.

Non-specific immunity was obtained by a single intraperitoneal injection of 100 to 200 μg. of purified *Salmonella* endotoxin in 0.2 ml. of saline administered 7 days prior to challenge.[2] The endotoxin was either freshly dissolved in saline the day of injection, or was stored in distilled water at 4°C. in a concentration of 10 mg. per ml. and was diluted to the desired concentration in 0.85 per cent saline. Lots kept at 4°C. as long as 8 months did not appear to lose their immunizing capacity. In 5 of the 6 experiments in which endotoxin was administered, the mice became sick within 24 hours of inoculation. From 0 to 40 per cent of mice in each group died during the first 7 days following endotoxin injection. Survivors appeared well at the time of challenge with virulent cryptococci. In the sixth study, all mice remained well between endotoxin administration and subsequent challenge.

Challenge Schedules.—

In each study, groups of immunized or control mice totaled 9 to 15 animals. Cryptococcus challenge consisted of 2.5×10^6 cells of virulent strains 1148 or 1149. Cells were harvested in saline from Sabouraud's agar slants incubated at 30°C. for 24 hours. The inoculum size was determined by direct hemocytometer count. One-half ml. was injected intravenously through a lateral tail vein.

Mice immunized with cryptococci were also challenged with one of three bacteria to determine the specificity of the immunity.

(*a*) *Staphylococcus aureus*, strain AZ. This strain of hemolytic *Staphylococcus aureus* was recovered from the lungs of a patient dying with staphylococcal pneumonia complicating Asian influenza in 1957 (13). It was coagulase-positive, fermented mannitol and was lysed by bacteriophages 53 VA4.

The culture was centifuged at 4,500 R.P.M. for 30 minutes after 24 hours growth in beef

[2] Lots AE 1298 S_4 and AE 1688 S_4 of pyrexal® were kindly supplied by Dr. Russell Schaedler of The Rockefeller Institute.

heart infusion broth at 37°C. The sediment was resuspended in physiologic saline in ⅔ the original volume. One-fifth ml. was inoculated intravenously *via* a lateral tail vein. This inoculum, which was approximately 10^8 staphylococci per mouse, was highly virulent for normal mice, killing 80 to 100 per cent of animals within 14 days (14).

(*b*) *Klebsiella pneumoniae*, strain Melton. This strain of Friedländer's bacillus which was isolated from a human infection was obtained from Dr. Peter Dineen of The New York Hospital. The strain has remained highly virulent for mice during serial agar passage. All control mice died within 7 days after the intravenous injection of 0.2 ml. of a 10^{-2} dilution of a 24 hour infusion broth culture incubated at 37°C.

(*c*) *Mycobacterium tuberculosis*, strain H37Rv. The history of this standard laboratory strain of mouse-virulent, human type tubercle bacillus has been thoroughly reviewed (15). When 0.2 ml. of a culture, grown 10 days at 37°C. in tween 80–albumin media, was injected intravenously, all control mice died within 2 months.

Period of Observation.—

Groups of mice challenged with cryptococci were observed for 3 to 6 months. The experiment was then terminated. In some experiments 4 or 5 survivors from each group were sacrificed for determination of tissue fungus populations. Mice challenged with *Mycobacterium tuberculosis*, *Klebsiella pneumoniae*, and *Staphylococcus aureus* were observed until all control and immunized mice were dead.

Determination of Tissue Populations.—

Control and immunized mice were sacrificed for determination of cryptococcus census in various tissues at several intervals during the first 14 days of challenge with 2×10^6 cells. Tissue populations were also determined in immunized mice surviving for 3 months after challenge with this inoculum.

Animals were killed by etherization. Liver, lungs, brain, or both kidneys were removed aseptically and were emulsified in 2 ml. of distilled water with a teflon tissue homogenizer (16). In some studies liver and spleen were emulsified together.

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. No attempt was made to separate strains 1148, or 1149, from strain B-27 in experiments in which the latter was used for immunization since it was found that the tissue populations of strain B-27 were small at the particular time each organ was studied and did not contribute significantly to the total fungus count. Futhermore, strain B-27 grew slowly in agar so that colony counts made at 2 to 5 days consisted exclusively of the more rapidly growing virulent strains 1148, or 1149.

Studies with Small Inocula.—

Twenty-seven normal mice were infected intravenously with 10^3 cells of strain 1149. The inoculum was harvested in saline from a Sabouraud's agar slant incubated at 30°C. for 24 hours. The number of cells in the suspension was determined by direct hemocytometer count and appropriate dilutions were made in saline. One-half ml. of a suspension containing 2×10^3 cells was inoculated into each mouse through a lateral tail vein. The exact size of this inoculum was determined by making pour plates of serial 10-fold dilutions of the injected suspension. Pour plates were incubated at 30°C. and colony counts were made at 2, 4, and 7 days.

Lung, kidney, brain, and combined liver-spleen populations were determined in groups of mice 1 day, 1 week, 1 month, 3 months, and 6 months after infection by techniques described above.

Statistical Analysis.—

The effect of immunization was studied by analyzing the cumulative mortality curves and by analysis of 3 month survivorship in control and immunized groups. The time of deaths following challenge was plotted on logarithmetic probability paper. The median time of death (ET50) and significance of protection were calculated according to the method of Litchfield and Wilcoxon (17, 18). Standard "+" tests were also performed. The significance of survivorship in immunized animals as compared to controls was studied by the method of chi square. The 3 month interval was chosen because mice that survived 90 days rarely died thereafter from cryptococcosis.

EXPERIMENTAL

1. Immunization with Intact Cryptococcus Cells.—

Modification of lethal cryptococcosis was readily demonstrated when mice were immunized with intraperitoneal or intravenous injections of intact cells of *Cryptococcus neoformans* and were subsequently challenged with 2.5×10^6 cells of a virulent strain. A single intraperitoneal injection of 2.5×10^4 cells of strain 1149, or virulent or avirulent strain 1148, a single intravenous inoculation of 2.5×10^6 cells of avirulent strain 1148, or three intraperitoneal injections of 2×10^7 cells of small capsule strain B-27, all appeared equally effective in protecting against challenge with virulent strains 1148 or 1149.

The results of 2 of the 8 studies are shown in Fig. 1 and 2. In both of these experiments the degree of prolongation of life produced by immunization with homologous strains 1148 or 1149 or heterologous strain B-27 was significant at the 1 per cent level.

Each of the 8 experiments performed is summarized in Table I. It may be seen that the severity of infection and degree of modification by immunization varied considerably. The time of survival of 50 per cent of control mice ranged from 16 to 37 days in 6 experiments. In one study it was only 6 days and in one, when strain 1148 had diminished in virulence, 50 per cent of controls lived for 52 days.

When the homologous strain was used for immunization, prolongation of life was significant at the 0.01 level in 4 of 5 studies and at the 0.05 level in the fifth experiment. When heterologous strain B-27 was used protection was significant at the 0.01 level in 3 experiments, at the 0.05 level in 5 studies.

Three month survival was also increased in the immunized animals. Nine of 93 control mice (9.7 per cent) survived the 3 month experimental period. Fifty-two of 138 (37.7 per cent) of mice immunized with strains 1148, 1149, or B-27 survived a similar period. The difference in survivorship is significant at the 1 per cent level.

There was no evidence in these studies that immunity was strain-specific. Thus immunization with large capsule strains 1148 and 1149 or small capsule strain B-27 produced similar protection to challenge with strains 1148 or 1149.

Immunization with *Cryptococcus neoformans* var. *innocuous*, strain JN, produced less protection. In each of the three experiments in which this strain

FIG 1. Effect of immunization on mortality in mice following intravenous challenge with 2.5 × 10^6 cells of *Cryptococcus neoformans*, strain 1148. Immunization schedules consisted of 3 intraperitoneal injections of 2 × 10^7 cells of heat-killed strain 1148, small capsule strain B-27, or avirulent strain JN. Ten to 13 mice were in each group.

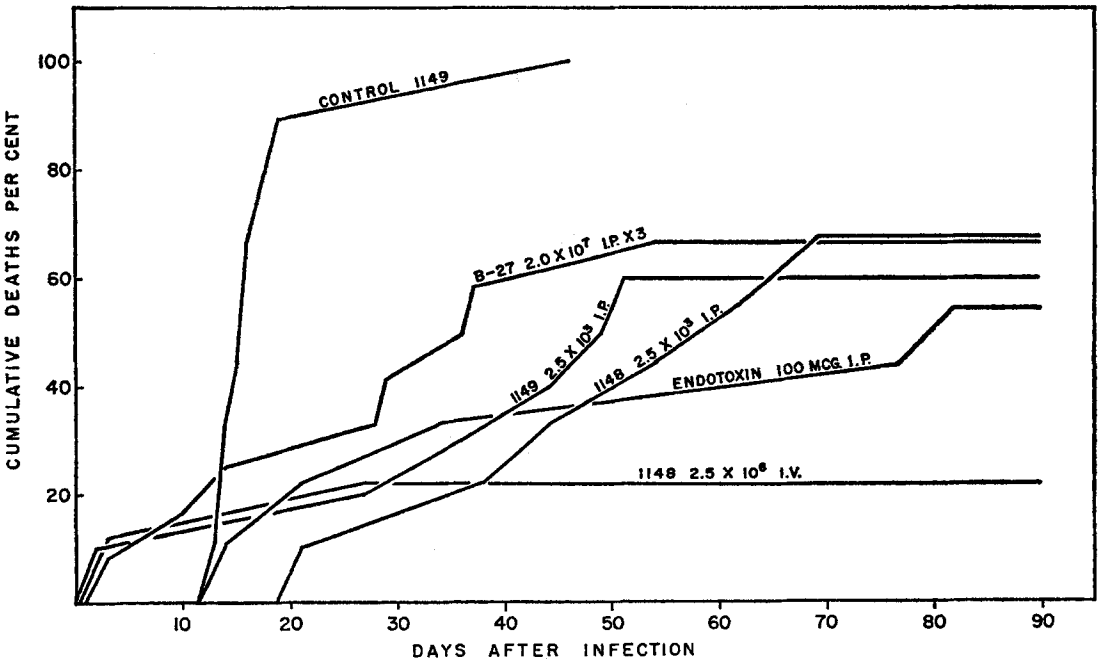

FIG. 2. Effect of specific and non-specific (endotoxin) immunization on mortality in mice challenged intravenously with 2.5 × 10^6 cells of *Cryptococcus neoformans*, strain 1149. Each group consisted of 9 to 12 mice. The endotoxin was administered 7 days prior to challenge.

was used the severity of the infection was modified, but in only one of these studies was the degree of prolongation of life statistically significant ($p < 0.05$). There was no increase in 3 month survival. One of the 3 individual experiments is included in Fig. 1 and all 3 are summarized in Table I.

Immunization with 3 intraperitoneal injections of heat-killed virulent strain 1148 produced no alteration in the course of lethal cryptococcosis (see Fig. 1 and Table I).

2. Immunization with Bacterial Endotoxin.—

Delay in death and increased 3 month survival was demonstrated in 5 of the 6 experiments in which 100 to 200 μg. of *Salmonella* endotoxin was injected intraperitoneally 7 days prior to intravenous challenge with 2.5×10^6 cells of *Cryptococcus neoformans* strains 1148 or 1149. The degree of prolongation of life was significant at the 0.01 level in 2 of the 5 studies and at the 0.05 level in the other 3.

Thirty of 60 (50 per cent) of endotoxin-treated mice survived the 3 month observation period as compared with 9 of the 61 controls (14.8 per cent) in these 5 studies. These differences in survivorship are statistically significant at the 0.01 level.

One of these 5 experiments is depicted in Fig. 2 and all 5 studies are included in Table I. It may be seen that protection following endotoxin administration was of similar magnitude to that observed after immunization with intact cryptococcus cells.

In each of the 5 experiments in which modification of infection was demonstrated the mice became sick after the injection of endotoxin. Zero to 40 per cent of the mice so inoculated died within 96 hours. The survivors demonstrated ruffled fur, and ate and drank poorly for a period of 48 to 96 hours. No correlation was noted between the severity of endotoxin sickness and subsequent protection. Thus, groups of mice which became sick but had no mortality from the endotoxin demonstrated the same degree of resistance to challenge that was noted in groups which became sick and had a 40 per cent mortality. All survivors appeared well at the time of cryptococcal challenge 1 week after endotoxin administration.

The mice were given the same lot of endotoxin in the sixth experiment. None became ill and no subsequent protection was observed. The failure of the endotoxin to produce illness or later protection has not been explained.

3. The Specificity of Immunity Induced with Intact Cryptococcal Cells.—

In three experiments (Experiments 4 to 6 Table I) mice were immunized with intact cryptococcus cells according to regimens detailed above which had been shown to produce significant modification of subsequent cryptococcal challenge. Groups of immunized mice were then challenged with *Staphylococcus*

TABLE I

The Effect of Immunization with C. neoformans and Bacterial Endotoxin on Survival Time and Survivorship Following Intravenous Challenge with 2.5 × 10^6 Cells of C. neoformans, Strains 1148 or 1149*

Exp. No.	Immunizing method	50 per cent survival	3 mos. survival Survivors/ infected	Degree of statistical significance of prolongation of life
		days		
1	Control	20	0/10	
	B-27 2 × 10^7 IP‡ × 3	33	0/9	0.01
	JN 2 × 10^7 IP × 3	27	0/8	0.05
	Heat-killed 1148 2 × 10^7 IP × 3	19	0/10	Not significant
2	Control	37	0/12	
	B-27 2 × 10^7 IP × 3	48	0/10	0.05
	JN 2 × 10^7 IP × 3	44	0/13	Not significant
	Heat-killed 1148 2 × 10^7 IP × 3	34	0/12	Not significant
3	Control	52	5/15	
	B-27 2 × 10^7 IP × 3	>104	10/15	0.05
	JN 2 × 10^7 IP × 3	>104	8/15	Not significant
	Endotoxin 100 μg. IP × 1	>104	13/15	0.01
4	Control	16	0/9	
	B-27 2 × 10^7 IP × 3	36	4/12	0.01
	1148 2.5 × 10^8 IP × 1	62	3/9	0.01

* IV, intravenous.

‡ IP, intraperitoneal.

TABLE I—*Concluded*

Exp. No.	Immunizing method	50 per cent survival	3 mos. survival Survivors/ infected	Degree of statistical significance of prolongation of life
		days		
4	1148 2.5 × 10^6 IV × 1	104	7/9	0.01
	1149 2.5 × 10^8 IP × 1	49	4/9	0.01
	Endotoxin 100 μg. IP × 1	66	4/9	0.01
5	Control	15	0/10	
	B-27 2 × 10^7 IP × 3	33	2/10	0.05
	1149 2.5 × 10^8 IP × 1	96	7/12	0.01
	Endotoxin 100 μg. IP × 1	13	0/12	Not significant
6	Control	6	0/12	
	B-27 2 × 10^7 IP × 3	67	5/11	0.01
	1148 2.5 × 10^6 IV × 1	13	2/8	0.05
	Endotoxin 150 μg. IP × 1	15	2/10	0.05
7	Control	18	1/13	
	B-27 2 × 10^7 IP × 3	32	6/14	0.05
	Endotoxin 200 μg. IP × 1	59	4/14	0.05
8	Control	20	3/12	
	B-27 2 × 10^7 IP × 3	40	2/10	0.05
	Endotoxin 100 μg. IP × 1	100	7/12	0.05

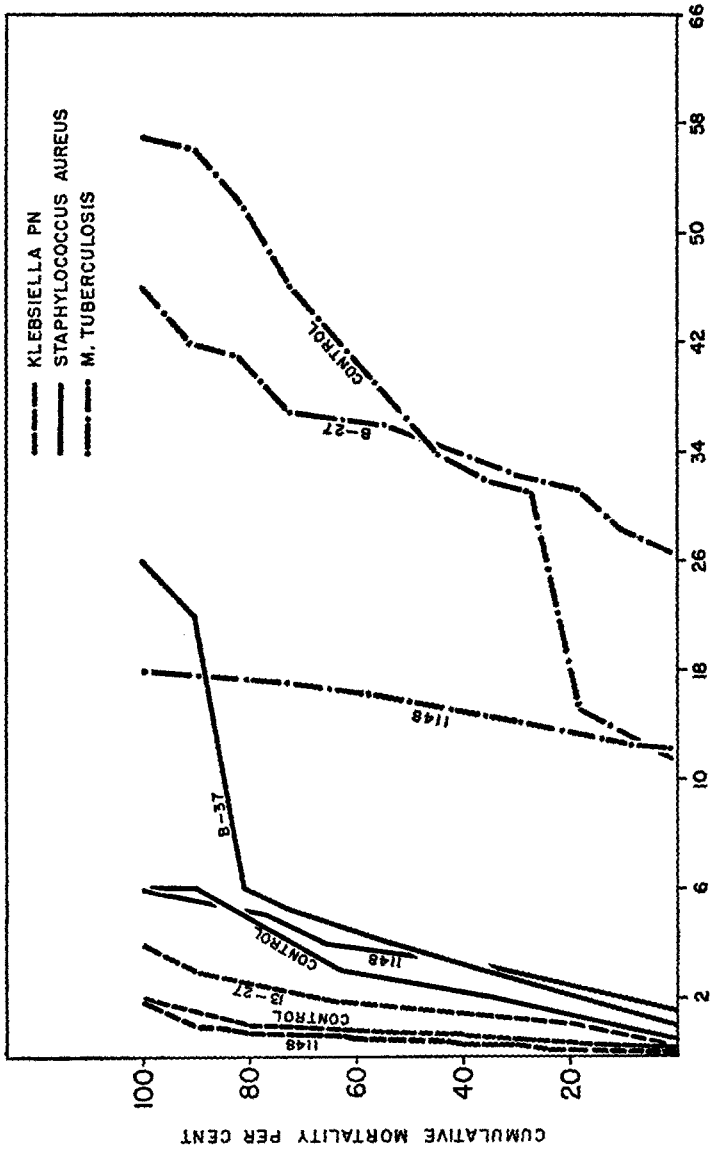

FIG. 3. The effect of immunization with cryptococci on heterologous challenge with *Staphylococcus aureus*, *Klebsiella pneumoniae*, and *Mycobacterium tuberculosis*. Eleven mice were in each group.

aureus, strain AZ, *Klebsiella pneumoniae*, strain Melton, or *Mycobacterium tuberculosis*, strain H37Rv. Each of the 3 microorganisms was also inoculated into a group of control mice. Mice immunized with cryptococcus cells were not significantly protected against challenge with staphylococci, klebsiellae or mycobacteria. Thus, immunized and control mice died at approximately the same rate following challenge with each of the three heterologous microorganisms. Indeed, when small numbers of virulent cryptococci were used in the immunization schedule, subsequent challenge with *M. tuberculosis* resulted in more rapid death than was observed in control mice infected with *M. tuberculosis* alone. Whether these deaths were due to enhancement of the cryptococcosis or the tuberculosis was not investigated.

A typical experiment is shown in Fig. 3.

The injection of intact cryptococcus cells appeared then to confer specific immunity. There was no evidence that such immunization protected against heterologous microbial challenge.

4. Tissue Population Studies Following Intravenous Injection of Large Numbers of Virulent Cryptococci.—

(a) Liver, lung, kidney, and brain populations in normal mice:

Initial trapping of fungi was maximum in the liver following intravenous injection of 2.5×10^6 cells of strains 1148 or 1149. Total number of cryptococci in the liver 4 hours after infection ranged from 1.2×10^5 to 4.2×10^5. Kidney, lung, and brain populations were on the average 6- to 100-fold lower at this time. Kidney and lung census ranged from 2.4×10^3 to 2×10^5 per organ. Populations were smallest in the brain and varied from 6×10^2 to 1.2×10^4 cells per brain.

Brain and liver populations did not change significantly over the first 24 hours. During the same period kidney and lung census fell an average of approximately $\frac{1}{3}$ and $\frac{2}{3}$ log unit respectively. A significant increase in fungus census as compared to that observed 24 hours after infection was noted in each of the 4 tissues studied 7 days after infection. Multiplication was slower in each organ between the 7th and 14th day of infection.

Maximum cryptococcus census was found in the brain at the end of both the 7 and 14 day periods. Brain cryptococcus populations varied from 7×10^6 to 5×10^7 7 days after infection. Lung and kidney census were generally somewhat lower at the end of the 1 week period ranging from 9.2×10^3 to 1.4×10^7 per organ. Multiplication was generally least marked in the liver. Liver census varied from 1.3×10^6 to 1.1×10^7 per organ.

During the 1st week of infection brain populations usually rose over 5000-fold. Lung and kidney census generally increased 50- to 500-fold. Liver populations rose only 10- to 50-fold.

These findings are summarized in Figs. 4 to 7.

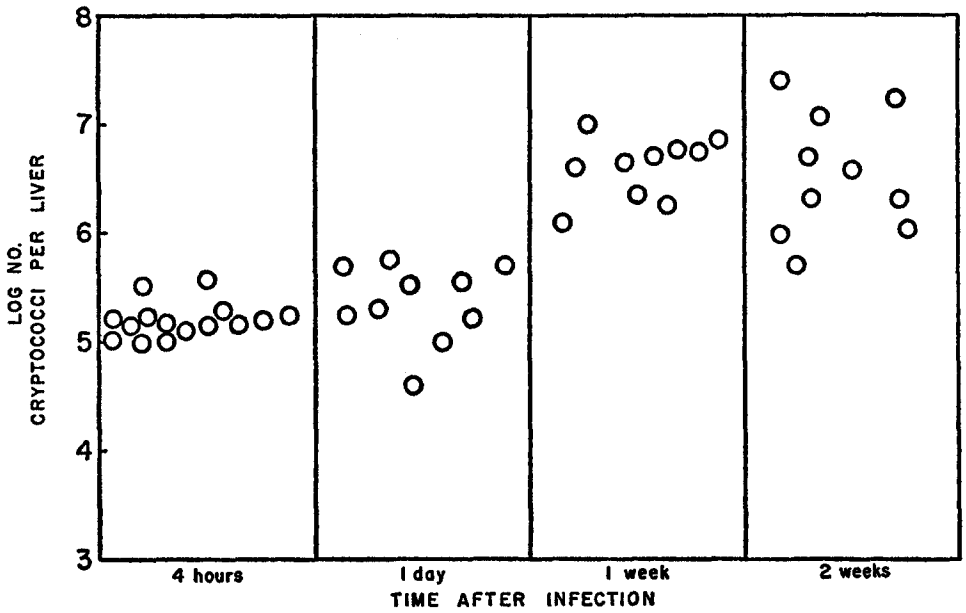

FIG. 4. Liver cryptococcus populations following intravenous infection with 2.5×10^6 cells of strain 1149.

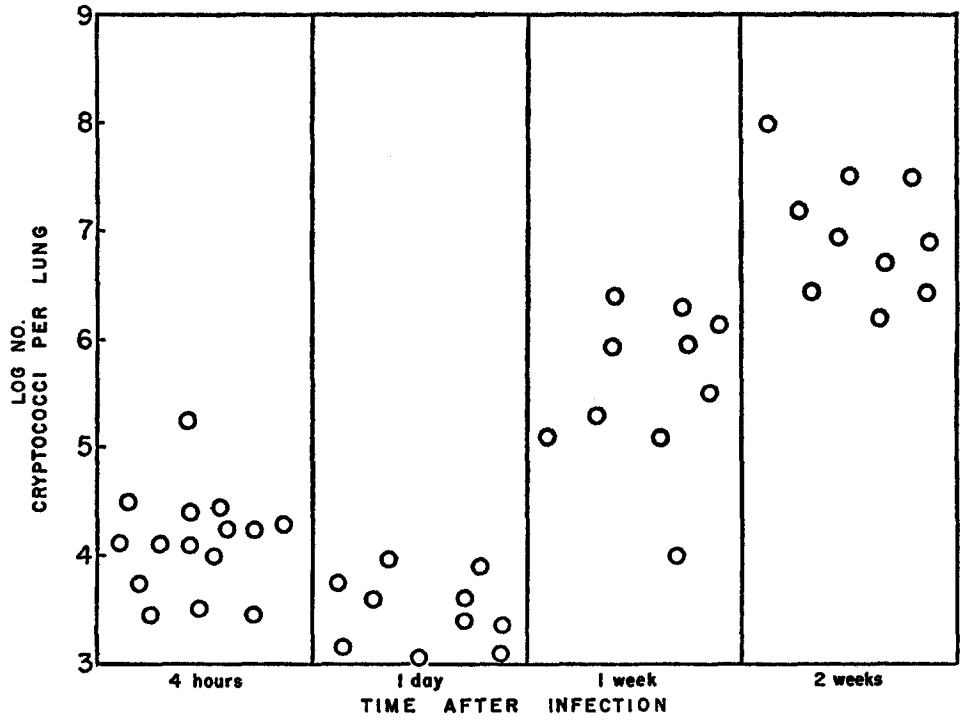

FIG. 5. Lung cryptococcus populations following intravenous infection with 2.5×10^6 cells of strain 1149.

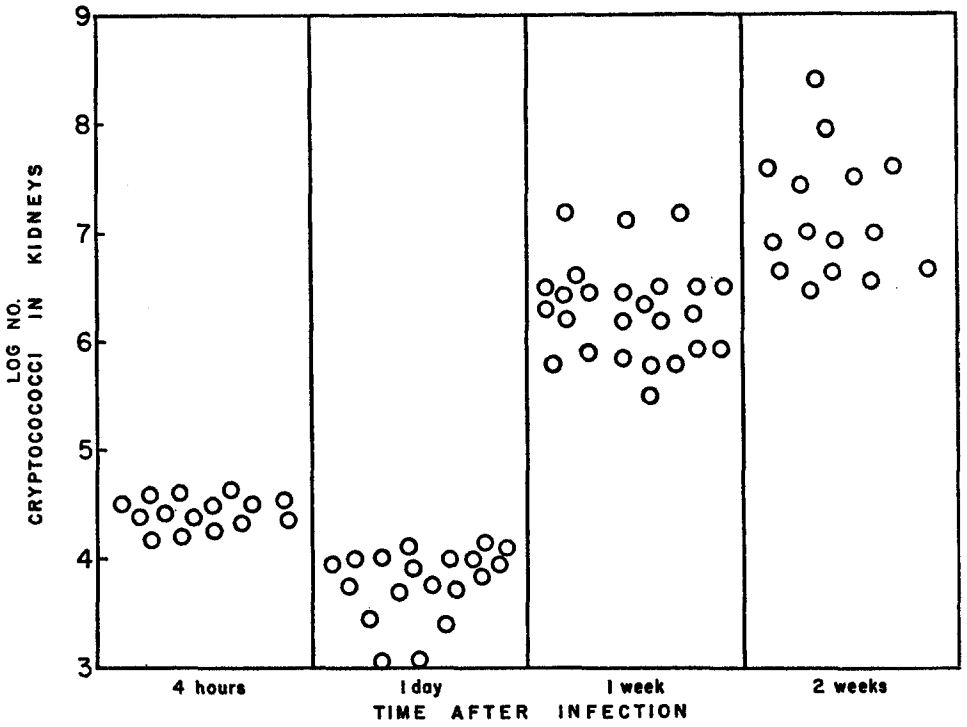

FIG. 6. Kidney cryptococcus populations following intravenous infection with 2.5×10^6 cells of strain 1149.

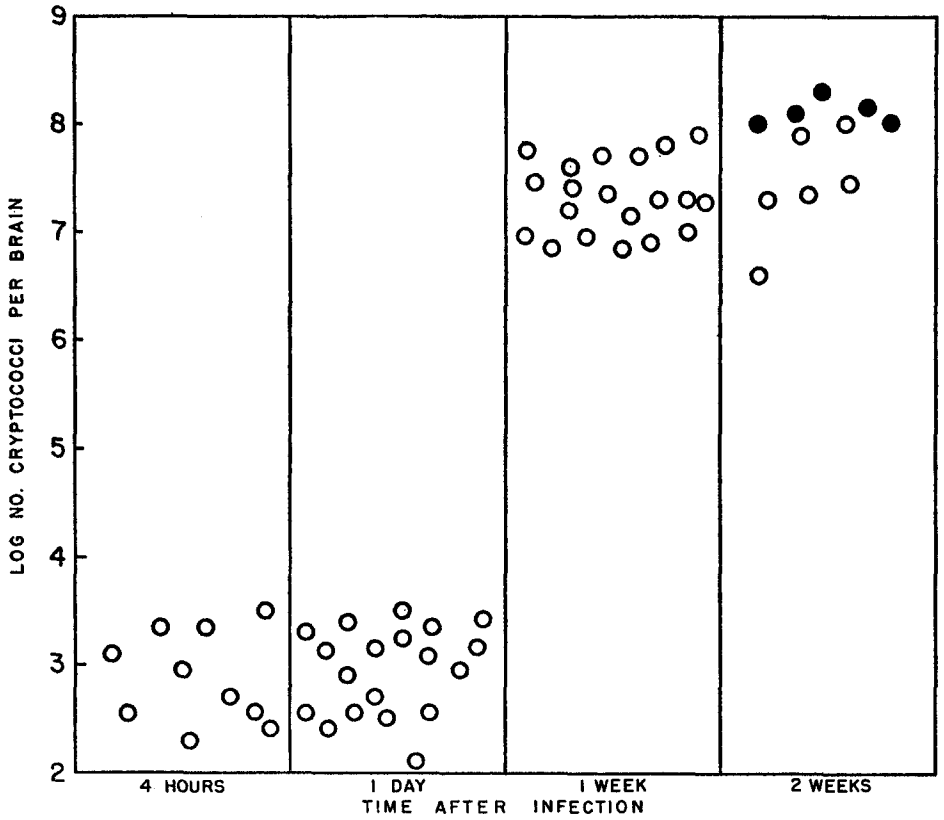

FIG. 7. Brain cryptococcus populations following intravenous infection with 2.5×10^6 cells of strain 1149. ●, mouse had gross hydrocephalus at time of sacrifice.

(*b*) Kidney and brain cryptococcus census in specifically and non-specifically immunized mice during the first 7 days of infection.

Three individual experiments were performed in which a total of 18 to 20 mice from each group were studied during the initial 24 hours of infection and 14 or 15 were studied 7 days after challenge. Results were similar in all 3 experiments and are recorded together. Strain B-27 was used for specific immunization in each of the 3 studies.

Populations in control and immunized mice were generally similar during the first 24 hours of infection. At the end of 7 days, however, striking differences were noted in tissue fungus census.

Brain populations were markedly smaller in 11 of 15 specifically immunized mice as compared to control animals. The cryptococcal census in the brains of these 11 animals 1 week after infection ranged from 2.7×10^4 to 3×10^5, an average increase of 38.3 times the titer found during the first 24 hours after challenge. Brain populations in control mice ranged from 7×10^6 to 5×10^7 7 days after infection, a mean increase of 5,550-fold. Brain titers for the whole group of 15 specifically immunized mice averaged 16-fold lower than values for control animals, a difference which is statistically highly significant ($p < 0.01$). On the contrary, brain populations in endotoxin-treated mice were only slightly (3.4-fold) smaller than those observed in control animals. The endotoxin-treated mice were, nevertheless, protected to the same degree as specifically immunized mice whose brain census 1 week after infection was markedly lower than control populations.

These observations are depicted in Fig. 8.

Findings in the kidneys were less striking. Cryptococcus census in specifically immunized mice averaged 5-fold less than controls 7 days after infection. This difference between the renal census in specifically immunized mice and in control animals was significant at the 0.05 level. Kidney populations in endotoxin-treated mice were generally somewhat greater than those observed for specifically immunized animals but averaged 2.7-fold lower than populations noted in control mice. There was considerable overlapping of values found for kidney census in endotoxin-treated mice and those observed in control or specifically immunized animals. The renal titers in endotoxin-treated mice were not statistically different from either controls or specifically immunized mice.

These observations are summarized in Fig. 9.

In summary, protection in specifically immunized mice was associated with a slower rate of fungus multiplication in the kidneys and brain during the first 7 days after challenge. Non-specific immunity, induced with bacterial endotoxin, was associated with similar protection to that observed with specifically immunized mice, but there was only slight inhibition of fungus multiplication in the brains and kidneys of these mice during the initial 7 days following challenge.

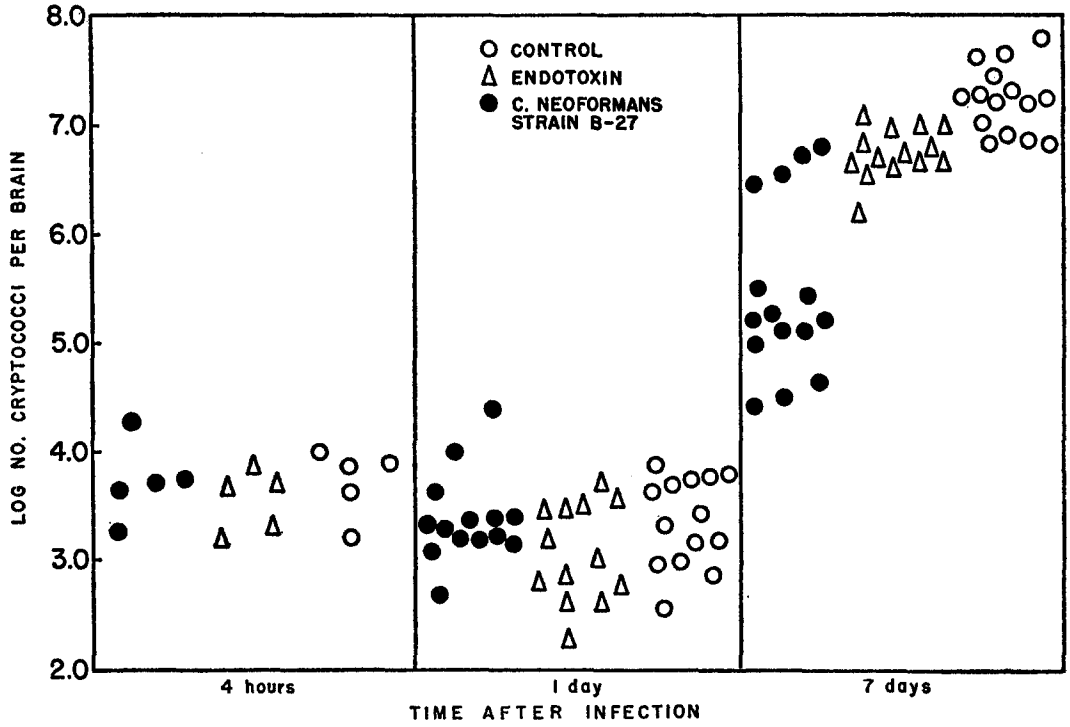

FIG. 8. Cryptococcus populations in the brains of specifically and non-specifically (endotoxin) immunized mice following intravenous challenge with 2.5×10^6 cells of strain 1149.

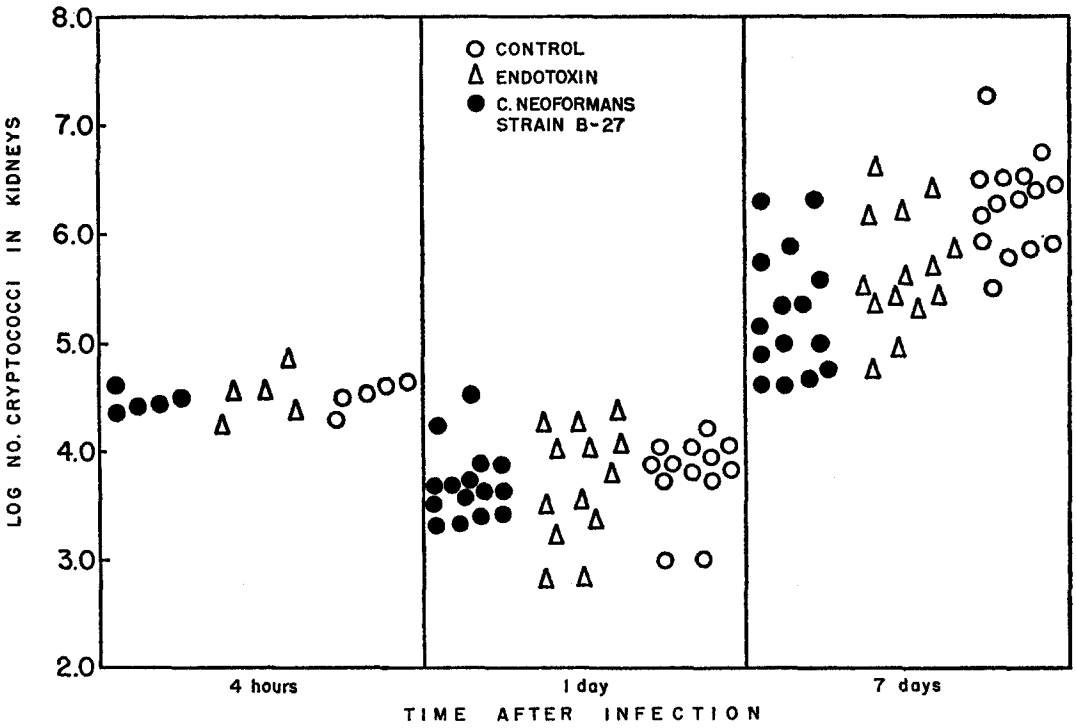

FIG. 9. Cryptococcus populations in the kidneys of specifically and non-specifically (endotoxin) immunized mice following intravenous challenge with 2.5×10^6 cells of strain 1149.

(*c*) Tissue populations observed in immunized mice 3 to 6 months after challenge.

Tissue cryptococcus census was determined in the liver-spleen, lung, brain, and kidneys of 4 or 5 mice from each of 4 groups surviving at least 3 months after challenge. These groups included 3 immunized specifically according to the different regimens previously described and one group immunized non-specifically with bacterial endotoxin.

Populations were generally markedly smaller than those observed in the organs of control mice dying 14 to 28 days after infection. These observations are recorded in Table II.

No significant differences were noted among the 4 groups of immunized mice studied.

Striking differences, however, were detected among the organs studied. Infection was most markedly diminished in the liver and spleen. In 11 of the 17 mice no cryptococci could be recovered from the combined liver and spleen. Persistent infection on the other hand was uniformly observed in the brain and kidney. Lung populations were intermediate. Five of 17 mice had no fungi cultured from the lungs at autopsy.

Thus clearance of infection was maximum in the liver, the organ of least multiplication in normal mice. Clearance on the other hand was less marked in the lungs and was least rapid in the brain and kidneys.

5. Tissue Populations in Normal Mice Following Intravenous Injection of Small Numbers of Virulent Cryptococci.—

Twenty-seven mice were inoculated intravenously with 10^3 cells of virulent strain 1149. Five mice were sacrificed for determination of liver, spleen, lung, kidney, and brain cryptococcus census, 1 day, 1 week, 1 month, 3 months, and 6 months after infection. Two mice died during the 6 month experimental period, one 5 weeks after, and the other 5½ months after infection. Tissue populations were also determined in these mice.

The results of this study are shown in Table III.

One day after infection, the cryptococcus populations were largest in the combined liver and spleen, the total number of fungus cells ranging from 2.4×10^2 to 8.0×10^2. Lung and kidney census was approximately 10-fold lower. Brain populations were smallest, and in 3 of the 5 mice studied, no cryptococci were detected.

During the first 7 days of infection progressive multiplication was observed in all tissues examined. Fungus census at this time ranged from 6×10^2 to 1.16×10^6 cells per organ. Multiplication was generally greatest in the brain and least in the liver and spleen.

During the next 6 months tissue populations fell progressively in most mice. Three months after infection, 3 of the 5 mice had virtually cleared their

TABLE II

Log No. Cryptococci in Organs of Immunized Mice Surviving 3 Months after IV Infection with 2.5 × 10^6 Cells of Strains 1148 or 1149

Method of immunization	Liver-spleen	Lung	Kidney	Brain
Strain B 27	0.30 0 1.42 3.18	0 1.53 0 3.18	4.81 4.53 1.87 4.55	4.34 3.26 3.38 5.30
2.5 × 10^7 IP × 3				
Strain 1149	6.12 0 0 0	6.09 3.16 0 0	6.03 1.91 2.81 2.0	2.62 2.85 3.62 1.26
2.5 × 10^8 IP × 1				
Strain 1148	1.51 0 0 0 3.0	2.68 3.0 3.08 0 6.48	5.79 1.30 3.60 2.55 4.78	3.56 3.68 2.82 1.81 4.60
2.5 × 10^6 IV × 1				
Endotoxin	0 0 0 0 0	4.25 3.42 3.56 0.78	1.53 5.08 3.15 2.21	3.08 3.82 3.68 3.75
100 μg. IP × 1				
No. sterile organs/ Total No. organs studied	11/17	5/17	0/17	0/17

TABLE III

Log No. Cryptococci in Organs of Mice after IV Infection with 10^8 Cells of Strain 1149

Time after infection	Liver-spleen	Lung	Kidney	Brain
1 day	2.86 2.45 2.38 2.60 2.90	0.90 1.15 0.60 1.83 1.60	1.45 1.75 2.38 1.66 0.78	0.60 0 0.78 0 0
1 wk.	4.23 5.38 4.58 4.03 5.30	3.26 4.63 3.92 2.78 4.80	4.85 5.18 3.93 4.41 6.06	3.78 3.90 5.45 5.87 5.26
4 wks.	2.32 2.47 2.15 0 0.60	2.36 2.78 2.30 0 0	3.73 2.61 3.20 2.66 1.82	3.78 6.02 5.48 0.90 1.70
5 wks.*	4.62	7.30	6.02	5.53
3 mos.	0 0 1.38 0 0	0.30 0 0.60 0 0	0 0.60 1.72 3.91 0	0 0 4.60 0 0
5½ mo.‡	1.42	1.85	1.15	5.30
6 mos.	0 0 0 0 0	0 0 0 0 0	0 0 0 0 0	0 0 0 1.68 0

* Animal expired.

‡ Sacrificed when moribund.

tissues of detectable cryptococci. Infection in the other two mice had also markedly diminished in most tissues, each having a single organ from which relatively large numbers of cryptococci could still be recovered.

Six months after infection, cryptococci were not detected in the tissue of 4 of the 5 mice. Small numbers of cryptococci (4.8×10^1) were found in the brain of the fifth mouse but other tissues were sterile.

Two of the 27 mice died with high tissue fungal census during the 6 month experimental period. In one dying 5 weeks after inoculation the lungs were the organ of the maximum infection. The other animal appeared well for 5 months, then became ill and died, 5½ months after infection. At autopsy, cryptococcus populations were largest in the brain.

DISCUSSION

These studies have demonstrated that:

1. The course of a lethal cryptococcus infection in mice could be modified by prior injection of small numbers of a virulent large capsule strain, or by injection of either small or large numbers of the same strain when it had decreased in virulence during serial passage on agar. Similar protection could also be produced by immunization with large numbers of a less virulent small capsule strain of *Cryptococcus neoformans*. Thus, immunization with one strain of *Cryptococcus neoformans* protected animals against challenge with a different strain. Immunization with *Cryptococcus neoformans* var *innocuous*, produced less modification of lethal cryptococcosis and injection with heat-killed cryptococci effected no protection. The induced immunity was species-specific and did not confer protection against heterologous microbial challenge with staphylococci, klebsiellae, or tubercle bacilli.

2. Modification of lethal cryptococcosis was also produced by the prior administration of bacterial endotoxin. It has been shown clearly that such immunity is non-specific and will confer protection against challenge with a variety of heterologous microorganisms (19, 20). The studies on non-specific resistance have not previously analyzed the effect of endotoxin administration on subsequent mycotic challenge.

3. Specific and non-specific immunity produced generally similar prolongation of life and approximately the same increase in 3 month survivorship.

4. The brain, the organ of maximum multiplication in normal mice during the first 14 days following intravenous injection of cryptococci, and the kidney, demonstrated the greatest incidence of persistent infection in immunized mice 3 to 6 months after challenge. The liver, the organ in which cryptococcus multiplication was smallest in normal animals, was the site of greatest clearance of the fungus 3 to 6 months after challenge.

5. The inoculation of small numbers of virulent cryptococci into non-imunized mice produced progressive disease in all tissues studied during the

early phase of the infection. Subsequently there was a marked decrease in tissue fungus census with eventual apparent eradication of the infection in most animals. In an occasional animal host defenses were inadequate and the mouse died with large tissue cryptococcal populations.

Although protection was of similar degree in specifically and non-specifically immunized mice, the mechanism of protection appeared to be distinctly different. In specifically immunized mice tissue multiplication of cryptococci was strikingly inhibited during the 1st week of infection. In mice non-specifically immunized with bacterial endotoxin, on the other hand, tissue multiplication was only slightly diminished during the first 7 days of infection.

The mechanisms of specific and non-specific immunity were not defined in this study. It seems reasonable, nevertheless, to assume that specific immunity is associated with the appearance of protective antibody. Agglutinating and precipitating antibodies have been clearly demonstrated in rabbits following immunization with cryptococci (7–9). Studies are currently in progress to determine whether agglutinating and/or cryptococcus-inhibiting antibody appears in the blood of specifically immunized mice. Studies are also being performed to determine whether specific immunity is associated with altered cellular response to cryptococcal invasion.

Two alternative explanations seem possible for the protection of endotoxin-treated mice in the absence of inhibition of fungus multiplication during the 1st week of infection. First it is possible that brain populations 14 days after infection, at the time controls began to die, would have been similar in endotoxin-treated and control mice and that endotoxin-protected mice did not die despite high cryptococcus titers. The administration of endotoxin produces profound physiological and biochemical alterations in the host (21, 22). Resistance is increased to later challenge with homologous and heterologous microorganisms, and the animal also becomes more resistant to the lethal effects of microbial toxins (23, 24). It is possible that death in cryptococcus-infected mice is related not only to the conncentration of cryptococci in tissues but also to undefined toxic substances. Salvin (25) has suggested that cryptococci do posses potentially lethal toxins. Endotoxin-treated mice might then be protected by their ability to resist toxic death despite high fungus census until specific host-immune mechanisms become effective.

This explanation seems less likely in view of the fact that, in most control mice, death appeared to be related to extensive hydrocephalus which occurred 14 to 28 days after infection. This was readily apparent upon inspection of the animal. Protection in endotoxin-treated mice was clearly associated with failure to develop hydrocephalus. It seems unlikely that endotoxin-treated mice would fail to develop hydrocephalus 14 to 28 days after challenge if brain populations were similar at that time to those observed in control animals.

The second alternative, therefore, appears more plausible. It seems quite possible that the endotoxin administration enhanced the immune response of the mouse so that antibody response was greater and/or more rapid following subsequent cryptococcus challenge. It has been shown that the simultaneous administration of small amounts of endotoxin and protein antigens markedy enhances the subsequent immune response to that antigen (26, 27). No increase in antibody response was observed in the aforementioned studies when polysaccharides were used as the antigen. The cryptococcus-agglutinating and precipitating antibodies in the rabbit appear to be related to the antigenicity of the capsule which is primarily polysaccharide in nature (5). Whether the protective antibody presumed to be present in immunized mice is also related to the capsular antigen is not known. It seems equally possible that such antibody is evoked by non-polysaccharide antigens in the yeast cell or cell wall.

If the mobilization of protective antibody were accelerated and/or increased so that significant antibody was present one week after infection in endotoxin-treated but not in control mice, the small decrease in renal and brain fungus census observed in endotoxin-immunized animals at that time would be explained.

It would then be anticipated that tissue cryptococcus census would be significantly smaller than control populations at the end of the 2nd week of infection at which time gross hydrocephalus begins to appear in controls but not in endotoxin-immunized mice. This hypothesis is under investigation at present as is a third possibility, namely that cellular defenses were altered in endotoxin-treated animals.

The ability of most mice to contain and eventually eradicate infection initiated with small numbers of cryptococci of strain 1149 appears to be of special significance. It seems likely in view of the early generalized progression of infection that host defenses in the non-immunized mouse are relatively poor. Adequate defenses are acquired in most mice after the 1st week. This sequence of events suggests that the acquisition of adequate defenses may be closely correlated with the development of specific antibody.

It should be emphasized that the findings herein reported concerning the acquisition of adequate defenses following infection with small inocula may not apply to certain other more virulent strains of *Cryptococcus neoformans*. Hasenclever and Mitchell (28) recently studied a strain of *Cryptococcus neoformans* which produced progressive infection and death following intravenous inoculation of 10^3 cells. It seems entirely possible that growth within the brain was more rapid during the 1st week of infection with this strain so that subsequent antibody production may have been ineffective is preventing the observed hydrocephalus.

It is of course uncertain as to whether the findings in these experiments in mice can be applied to human torulosis. In both man and mouse the central

nervous system is clearly the tissue of maximum infection (1). Littman (29) has suggested that the central nervous system is the site of maximum cryptococcal census in man because the thiamine content of cerebrospinal fluid is optimum for cryptococcal capsular formation.

The results of these studies suggest the following alternative thesis.

1. Infection is initiated with small numbers of cryptococci. In man the site of invasion is commonly in the respiratory tract (1).

2. Early cellular and humoral defenses are poor so that progressive local infection ensues, accompanied frequently by fungemia.

3. Increase in fungus census occurs in all tissues involved by fungemic spread. Subsequent limitation and eradication of infection is then dependent primarily on the production of specific antibody. In patients whose immune mechanisms are deranged, progressive infection in many tissues is likely to follow cryptococcal invasion. Clinical evidence offers some support for this thesis since widespread visceral cryptococcosis is limited almost exclusively to patients with severe underlying disease of the reticuloendothelial system (30) whose immune mechanisms are clearly deficient (31, 32).

4. Central nervous system invasion follows fungemia in some patients. Here as in other tissues, control of infection is dependent on the development of specific antibody. Although the blood-cerebrospinal fluid barrier is markedly deranged in cryptococcosis[3], it is unlikely that the antibody concentration would be as large as that in blood. Infection in the central nervous system would then be less well controlled, thus establishing this as the site of maximum fungus census in the later stages of the disease.

SUMMARY

The course of lethal cryptococcosis in mice was modified by immunization with the same strain or different strains of *Cryptococcus neoformans*. Protection was associated with a definite decrease in tissue fungus multiplication over the initial 7 days of infection. Such immunity was species-specific and did not protect against heterologous challenge with *Staphylococcus aureus*, *Klebsiella pneumoniae*, or *Mycobacterium tuberculosis*.

Similar modification of lethal cryptococcosis was produced by intraperitoneal inoculation of 100 to 200 μg. of *Salmonella* endotoxin 7 days prior to challenge. In non-specifically (endotoxin) immunized mice there was only a minimal reduction in tissue cryptococcus census during the 1st week of infection.

When small numbers of virulent cryptococci were injected intravenously into normal mice, tissue populations rose markedly in all organs studies during

[3] Unpublished studies by the author have demonstrated that the blood-cerebrospinal fluid bromide ratio falls from the normal 2.5 to 3.0:1 to 1:1 in cryptococcal meningitis in man. These findings are similar to those found in tuberculous meningitis (33) and indicate a marked alteration in the blood-cerebrospinal fluid barrier.

the first 7 days. A progressive fall in tissue fungus census was then observed in most mice during the next 6 months.

The increase in resistance to cryptococcal challenge following specific immunization, the non-specific protection conferred by injection of bacterial endotoxin and the ability to control and eventually reduce infections with small inocula may well be dependent in each case on the development of specific antibody.

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