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Immunization of Mice by Intracutaneous Inoculation with Viable Virulent *Cryptococcus neoformans*: Immunological and Histopathological Parameters

STEPHEN A. MOSER,[1,2]†* FRANK L. LYON,[1] JUDITH E. DOMER,[1] AND JAMES E. WILLIAMS[2]

Department of Microbiology and Immunology, School of Medicine, Tulane University, New Orleans, Louisiana 70112,[1] and Department of Pathology and Laboratory Medicine, Mount Sinai Medical Center, Milwaukee, Wisconsin 53201[2]

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Immune responses, including protection and delayed hypersensitivity, were evaluated in experimental murine cryptococcosis. Mice were immunized by the intracutaneous inoculation of viable virulent *Cryptococcus neoformans* yeasts. Response to the cutaneous infection was evaluated histologically and by cultural assays of the internal organs, as well as by intravenous challenge with the same strain. Protection was assessed by survival, histopathology, and quantitative organ culture. The intracutaneous inoculation of cryptococci resulted in a local inflammatory response that effectively limited dissemination of the organisms systemically and induced the development of delayed hypersensitivity demonstrable with a membrane extract of *C. neoformans* and with soluble cytoplasmic substances. A protective response was induced by the cutaneous inoculation of cryptococci as well, in that immunized animals survived longer, with about 25% of the challenged group ridding themselves completely of the cryptococci. Protection could be demonstrated by cultural analyses, but all animals, whether control or immunized, allowed considerable multiplication of the inoculum during the first 4 weeks after intravenous challenge. It would appear, therefore, that the protective mechanism(s) required additional antigenic stimulation before it could eventually function to eliminate all cryptococci from tissues. Histologically, there were no differences in pathology of the internal organs between immunized and unimmunized animals. Although the model described herein for the induction of immune responses in murine cryptococcosis has at least one drawback, viz., the presence of cryptococci in the skin lesion of many animals throughout the duration of the experiment, it does have the advantage that the immune responses were stimulated by a virulent strain and only minimal dissemination occurred. Therefore, lymphocytes could be removed from animals that were not contaminated with cryptococci for in vitro and in vivo transfer.

The nature of protective immunological responses in cryptococcosis is not yet completely defined, although experimental evidence indicating a major role for T-lymphocytes has accumulated in recent years (2, 5, 13–16, 22, 30). Mice have been the primary animals used in laboratory studies, and investigators have used various approaches to explore immune responses to *Cryptococcus neoformans*. For example, some investigators (1, 17, 26) have attempted to stimulate protection by immunization with killed whole cells or cell fractions, with or without adjuvant, whereas others (8, 23, 25, 26) have used live cells administered intravenously (26), intraperitoneally (23, 25), intranasally (23, 40), or subcutaneously (8). Viable cells administered intraperitoneally (23, 25) or via the pulmonary route (23, 40) disseminated rapidly. Another approach has been to immunize with avirulent mutants and challenge with the virulent wild type (11, 35). In general, viable cells have appeared to be more effective than nonviable cells, but often the degree of protection conferred by immunization, regardless of the nature of the immunogen, has been minimal. Furthermore, the study of immune responses other than protection in the sensitized animals has been hampered by the lack of suitable antigens for in vivo and in vitro assays. We report here our own approach to the study of immune responses in experimental murine cryptococcosis, wherein mice were immunized by the intracutaneous

† Present address: Department of Pathology and Laboratory Medicine, Mount Sinai Medical Center, Milwaukee, WI 53201.

inoculation of viable virulent cryptococci and the response to infection was monitored histologically, culturally, and by assays for protection and cell-mediated immune responses.

MATERIALS AND METHODS

Organism. *C. neoformans* 145 (serotype A), originally isolated from a case of human cryptococcosis at Tulane University School of Medicine, has been maintained in our stock culture collection. Its identification was verified by characteristic morphology, assimilation pattern (24, 28), production of urease (28), and pigment formation on bird seed agar (38). Stock cultures were maintained at 4°C on Sabouraud dextrose agar (Difco, Detroit, Mich.) and transferred monthly.

Animals. Ten to twelve-week-old male CBA/J mice purchased from Jackson Laboratories, Bar Harbor, Maine, were used in all experiments.

Preparation of inocula and inoculation of mice. *C. neoformans* was grown in unmodified Trypticase soy dialysate broth (36) at 37°C at 160 rPM in a gyratory shaker incubator. After 72 h of incubation, the culture was centrifuged at 10,000 $\times g$ for 10 min, and the organisms were washed three times in sterile nonpyrogenic saline before resuspension in the same diluent. Serial 10-fold dilutions were prepared, and viable cell counts were determined by plating on Sabouraud dextrose agar and incubating at 37°C for 48 h. The washed inoculum was held at 4°C while viability was being determined; it was then diluted to contain the desired number of viable cryptococci in 0.05 ml if used to immunize mice via the cutaneous route or 0.5 ml if used for intravenous challenge. The cutaneous inoculation was administered on the shaved flank, and the intravenous challenge was administered in the lateral tail vein.

Preparation of antigens. *C. neoformans* was grown in Trypticase soy dialysate which had been modified by deletion of the vitamin supplementation, addition of 2.9% (wt/vol) NaCl (9), and adjustment of the pH to 5.0 (9). Flasks (500 ml) containing 100 ml of modified Trypticase dialysate broth were inoculated with 5.0×10^8 cryptococci as determined by hemacytometer counts and incubated for 24 h at 30°C at 160 rPM in a gyratory shaker incubator. Blastospores were harvested by centrifugation and washed in Tris buffer containing 0.02 M $MgCl_2$ (6) until the supernatant no longer reacted in the phenol-sulfuric acid assay for polysaccharides (7). Packed, washed cryptococci were resuspended in buffer to a cell/buffer ratio of 1:2 and disrupted by ballistic action in a Braun MSK cell homogenizer for 8 min, and the homogenate was separated into several fractions by differential centrifugation (Fig. 1). Soluble cytoplasmic substances (SCS) were dialyzed against sterile distilled water, lyophilized, and stored in vacuo over desiccant. The membrane-mitochondria fraction was washed in sterile nonpyrogenic saline, dialyzed against sterile distilled water, and extracted for proteins as previously described (31) to yield an extract referred to as B-HEX. After dialysis to remove $(NH_4)_2SO_4$, the B-HEX was stored at −20°C. Carbohydrate was estimated by the phenol-sulfuric acid assay (7), and protein was measured by the method of Lowry et al. (27).

Quantitative culture of tissues. Each organ or lesion to be cultured for cryptococci was removed from mice after exsanguination via cardiac puncture while under ether anesthesia, homogenized in toto in saline, diluted, and plated onto Sabouraud dextrose agar containing chloramphenicol. The numbers of colony-forming units (CFU) per organ were calculated, and the geometric means of $\log_{10}$ values were determined for use in statistical analyses.

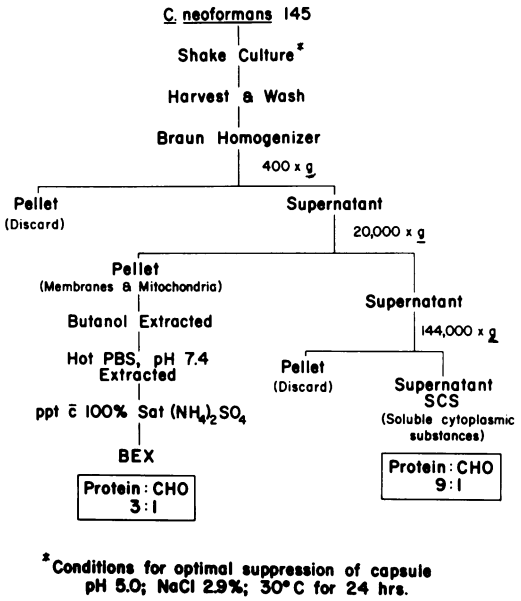

FIG. 1. Flow chart for the preparation of antigens used in testing for delayed hypersensitivity.

Footpad testing. Mice were footpad tested with B-HEX (20 μg of protein; 7 μg of carbohydrate per dose) or SCS (20 μg of protein per dose) as previously described (12, 31). Briefly, the test antigens were prepared so that the appropriate concentration was contained in 0.02 ml of nonpyrogenic saline, which was delivered through a 30-gauge needle on a micrometer syringe (Gilson). Feet were measured with a spring-loaded dial micrometer calipers just before and 0.25, 4, 7, 24, and 48 h after injection. The mean net increase in footpad thickness was determined by subtracting preinjection values from postinjection values.

Histology. Tissues were fixed in 10% Formalin, embedded in paraffin, cut into sections of 5-μm thickness, and stained with either hematoxylin and eosin (H&E) or periodic acid-Schiff (PAS) by standard methods (29).

Statistical analyses. All data were analyzed for statistical significance by one-way analysis of variance comparing experimental with control observations for any given condition.

RESULTS

Characterization of the cutaneous inoculation of viable cryptococci. Groups of mice were inoculated cutaneously with 10^2, 10^3, or 10^4 viable cryptococci, and three mice from each group were sacrificed for culture at selected weekly

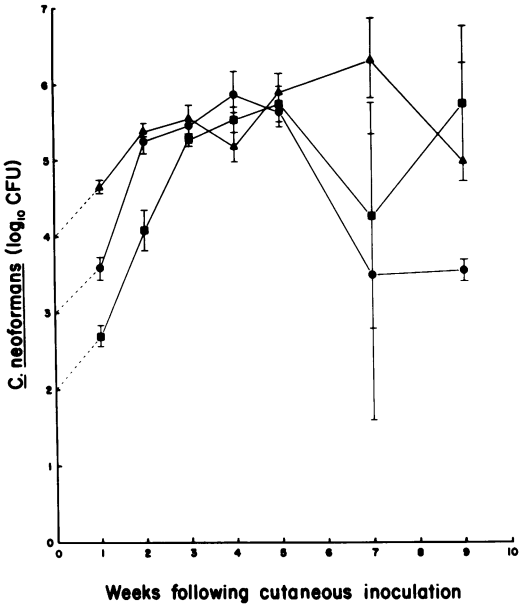

FIG. 2. Persistence of *C. neoformans* at the cutaneous inoculation site ($n = 3$) after the injection of 10^2 (■), 10^3 (●), or 10^4 (▲) viable cells.

intervals over a period of 12 weeks. The site of the cutaneous inoculation was cultured to determine survival in the skin, and the lymph nodes draining the site of the cutaneous inoculation, as well as spleen, lung, and brain, were cultured to assess systemic spread.

The intracutaneous inoculation of all doses of viable *C. neoformans* resulted in the establishment of a localized infection. The number of cryptococci in the lesions rose to 10^5 to 10^6 CFU, regardless of starting inoculum, by the 3rd week, and that level was maintained through the 5th or 6th week, depending on the experiment (Fig. 2). Infections in all groups were relatively uniform in the early weeks, but thereafter some animals eliminated the organism from the cutaneous site, whereas others did not, resulting in considerable variability in the quantitative data. All immunized mice developed a noticeable induration at the site of the cutaneous inoculation by the 2nd or 3rd week. The draining inguinal lymph nodes were visibly enlarged at this time as well and appeared to be two to three times larger than those from uninoculated animals.

Quantitative cultures of tissues other than skin (Tables 1 and 2) illustrated the fact that although some dissemination from the cutaneous site did occur, it was not frequent, and the numbers of cryptococci recovered in any given tissue were minimal. In only one instance was the number greater than 10^2 yeasts. The frequency of positive cultures was highest from 4 to 8 weeks after cutaneous inoculation, and no correlation between inoculum size and dissemination was observed.

Histological examination of cutaneous lesions 3 weeks after inoculation showed a localized, intradermal inflammatory reaction consisting predominantly of macrophages and monocytes with a few scattered neutrophils, lymphocytes, and multinucleated giant cells (Fig. 3). The number and distribution of the latter cells varied considerably among animals. Widely ranging numbers of both obviously encapsulated and apparently nonencapsulated *C. neoformans* were located within the central portion of the injection site. Some of the sites had large clusters of yeast cells, but in other injection sites there were few organisms, and PAS staining was necessary to demonstrate their presence.

Survival of unimmunized and cutaneously infected mice after intravenous challenge. To determine an appropriate dose for intravenous challenge of immunized mice, groups of 20 unimmunized mice were challenged with graded doses of cryptococci and monitored for survival (Fig. 4). The higher doses, viz., 10^3 and 10^4, killed animals too quickly and would likely have masked protective responses, whereas 10 cells, although resulting in deaths after a prolonged period, seemed too few and would have been too difficult to reproduce. Therefore, 100 cells was selected as the challenge dose for subsequent experiments.

To assess protection after cutaneous inoculation, groups of 20 mice were inoculated cutaneously with 10^2, 10^3, or 10^4 viable cryptococci and then challenged intravenously with 10^2 viable cryptococci 3 or 6 weeks thereafter. Immunization with 10^4 cells cutaneously resulted in 30

TABLE 1. Summary of systemic spread of viable *C. neoformans* over a 12-week period in mice infected cutaneously with 10^2, 10^3, or 10^4 viable *C. neoformans*

Organ	Expt I		Expt II		Combined	
	Positive/total	% Positive	Positive/total	% Positive	Positive/total	% Positive
Draining lymph node	2/61	3.3	7/72	9.7	9/133	6.8
Spleen	2/61	3.3	11/72	15.3	13/133	9.8
Lung	1/61	1.6	7/72	9.7	8/133	6.0
Brain	1/61	1.6	2/72	2.8	3/133	2.3
Total animals	6/61	10.0	14/72	19.4	20/133	15.0

TABLE 2. Systemic spread of *C. neoformans* after intracutaneous inoculation with varying doses of viable yeasts[a]

Week[b]	Infecting dose	Draining lymph node		Spleen		Lung		Brain	
		CFU[c]	No. positive	CFU	No. positive	CFU	No. positive	CFU	No. positive
1	10^2	0		0		0		0	
	10^3	0		0		0		0	
	10^4	0		0		0		0	
2	10^2	0		0		0		0	
	10^3	0		0		0		0	
	10^4	0		1	1	0		0	
3	10^2	0		0		0		0	
	10^3	0		60	1	0		0	
	10^4	0		0		0		0	
4	10^2	0		0		0		0	
	10^3	0		30	3	76	3	8	1
	10^4	74	1	2	1	0		0	
6	10^2	0		0		0		0	
	10^3	5	2	0		0		0	
	10^4	0		3	1	2	1	2	1
8	10^2	0		0		0		0	
	10^3	5	2	29	2	9	1	0	
	10^4	0		0		0		0	
10	10^2	0		0		0		0	
	10^3	0		0		0		0	
	10^4	10	1	3	1	3	1	0	
12	10^2	14	1	114	1	3	1	0	
	10^3	0		0		0		0	
	10^4	0		0		0		0	

[a] The organs from three animals were cultured quantitatively for each dose and observation time.
[b] After cutaneous inoculation.
[c] Average per entire organ.

to 35% survival through 90 days whether challenged intravenously at 3 (Fig. 5) or 6 (Fig. 6) weeks after the cutaneous inoculation.

Immunization with 10^2 or 10^3 cryptococci cutaneously resulted in less of a protective response at both 3 (not shown) and 6 (Fig. 6) weeks. Twenty percent of the animals immunized with 10^4 cryptococci cutaneously 3 or 6 weeks before intravenous challenge, or with 10^3 cryptococci 6 weeks before challenge, were culturally negative 90 days after intravenous challenge. All animals inoculated cutaneously with 10^2 cryptococci before intravenous challenge were culturally positive in one or more tissues 90 days after intravenous challenge. On the basis of these data, 10^4 cryptococci were inoculated cu-

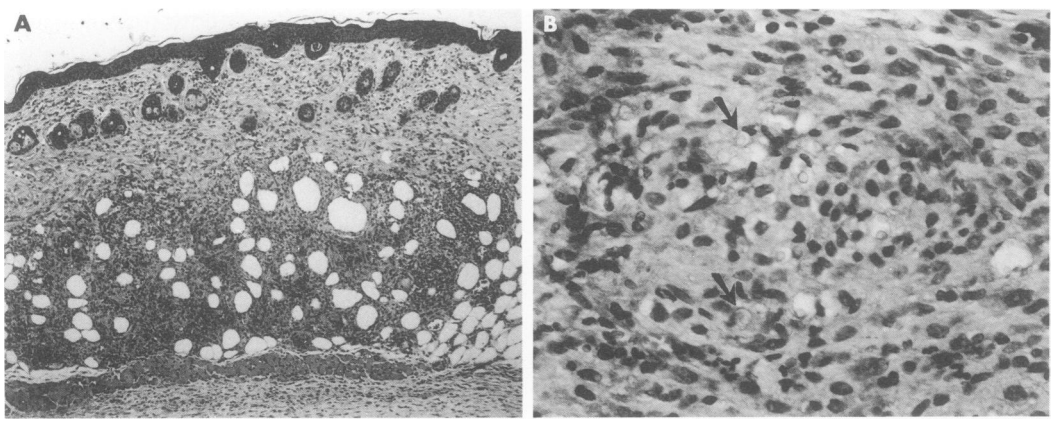

FIG. 3. (A) The cutaneous inoculation site 3 weeks after the deposition of 10^4 viable *C. neoformans* shows a well-defined lesion (×63, H&E). (B) The granulomatous reaction consists of macrophages containing phagocytized cryptococci (arrow) (×220, H&E).

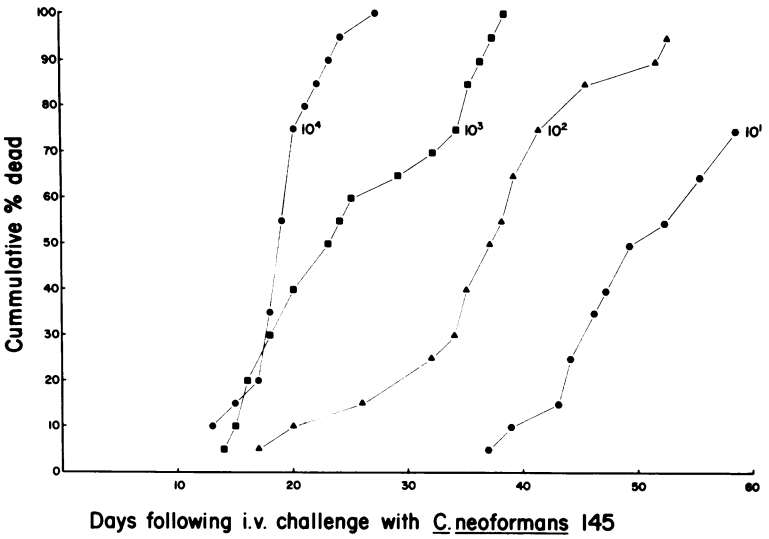

FIG. 4. Survival of unimmunized mice (n = 20) after an intravenous challenge with 10^1, 10^2, 10^3, or 10^4 viable *C. neoformans* cells.

taneously as the immunizing dose in all subsequent experiments.

Histopathology after intravenous challenge of mice immunized by the cutaneous inoculation of viable cryptococci. Uninfected mice as well as mice which had been infected cutaneously 3 weeks before challenge were challenged intravenously with 10^2 viable virulent cryptococci and sacrificed at selected intervals thereafter through 6 weeks for histological examination of brains, lung, liver, spleen, and kidney. Since there were no histopathological differences between immunized and unimmunized mice, the following descriptions pertain to both groups.

Brains showed evidence of either marked meningitis (Fig. 7) or minimal meningitis with associated encephalitis (Fig. 8). A markedly widened subarachnoid space infiltrated by equivalent numbers of monocytes and neutrophils admixed with numerous encapsulated cryptococci was characteristic of marked meningitis. In contradistinction, mice with minimal meningitis had a slightly widened subarachnoid space which contained an inflammatory infil-

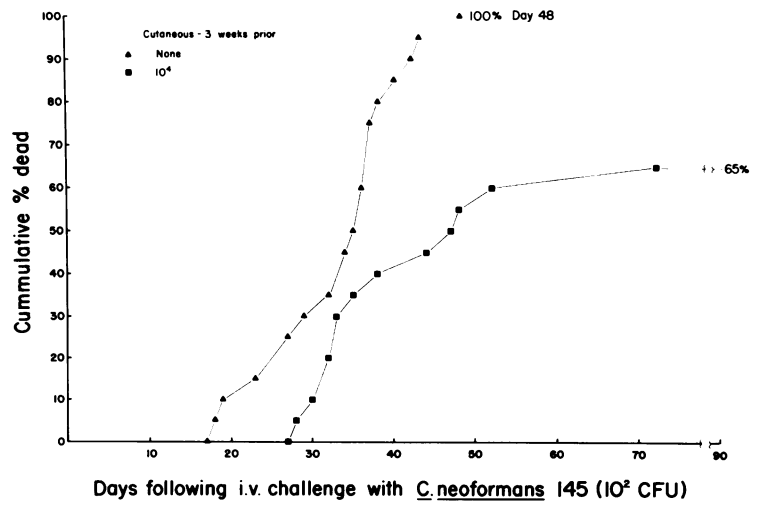

FIG. 5. Survival of unimmunized mice (n = 20) and mice immunized by the cutaneous inoculation (n = 20) of 10^4 of viable *C. neoformans* when challenged intravenously with 10^2 *C. neoformans* 3 weeks after the cutaneous inoculation.

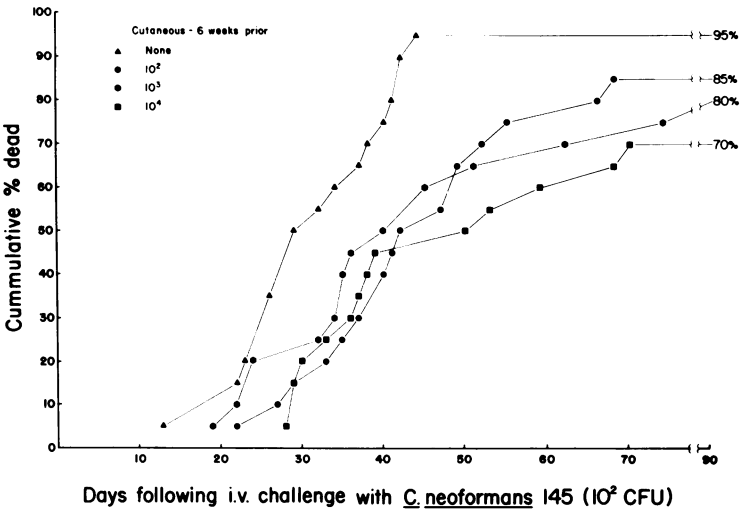

FIG. 6. Survival of unimmunized mice (n = 20) and mice immunized intracutaneously 6 weeks earlier with viable *C. neoformans* (n = 20) after intravenous challenge with 10^2 *C. neoformans*.

trate consisting mainly of monocytes, with a few neutrophils and lymphocytes which were admixed with considerably fewer encapsulated yeast cells than were present in the marked meningitis. Adjacent to foci of meningitis in some of the mice were cystlike intracerebral and intracerebellar cavities (Fig. 8A). The cavity was empty except for widely separated clusters of yeast cells (Fig. 8D). Little or no inflammatory infiltrate was present in the wall of most of the cavities (Fig. 8C). When present, inflammatory response consisted of a minimal lymphocytic, perivascular cuffing. Sections stained with PAS revealed individual, nonencapsulated yeast cells lying within the brain which were not apparent in the H&E sections. Within the cerebrum of one of the immunized mice there was a single granulomatous lesion (Fig. 9). Centrally it contained foamy histiocytes and scattered cryptococcal organisms which were slightly encapsulated. Reactive gliosis, foreign body giant cells, marked perivascular infiltrate of lymphocytes, and an occasional plasma cell were present in the periphery of the lesion.

Within the infected livers there were single to multiple inflammatory nodules (Fig 10). Lesions consisted of either neutrophils or neutrophils admixed with monocytes, macrophages, and lymphocytes in various proportions. Within lesions containing mainly monocytes, there were occasional foreign body giant cells. The few (one to four) encapsulated yeast cells which were

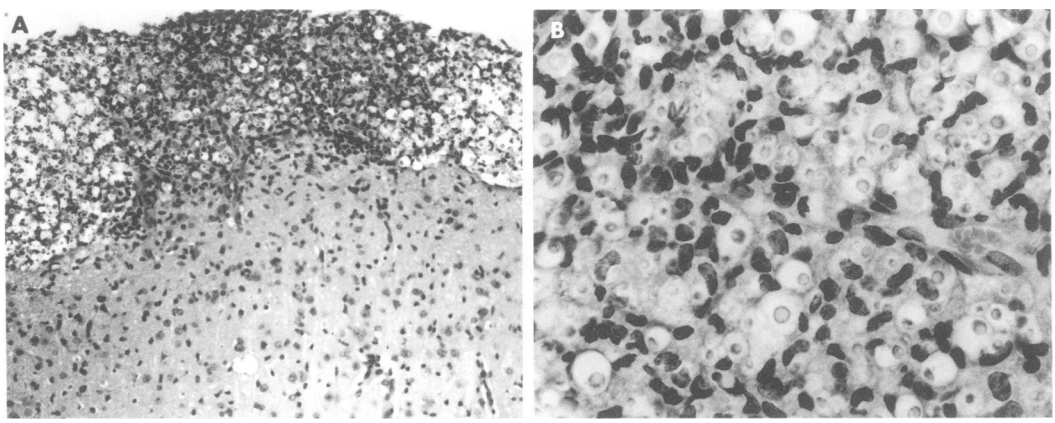

FIG. 7. (A) Brain from an immunized mouse 3 weeks after intravenous challenge demonstrates a severe meningitis (×88, H&E). (B) Numerous encapsulated cryptococci, monocytes, and scattered polymorphonuclear leukocytes are packed into the widened subarachnoid space (×352, H&E).

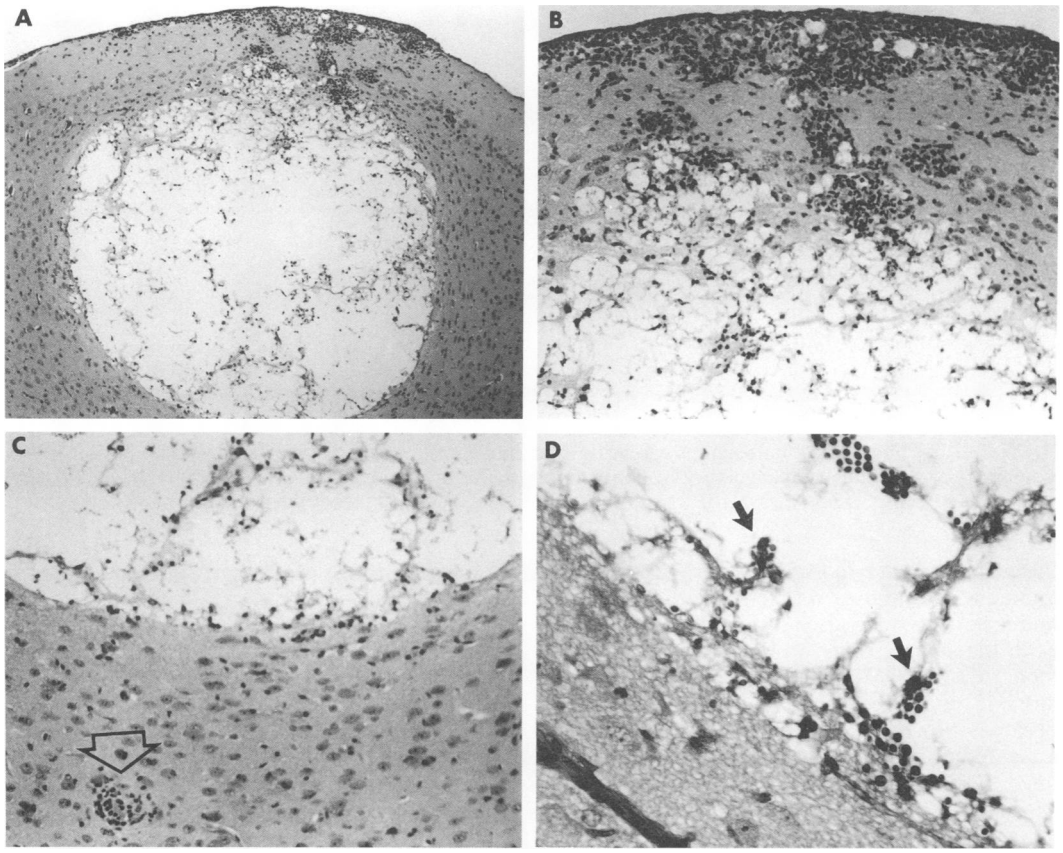

FIG. 8. (A) Focal, minimal meningitis with adjacent cerebritis from an unimmunized mouse 4 weeks after intravenous challenge shows a typical cystlike lesion (×63, H&E). (B) The subarachnoid space contains monocytes and scattered polymorphonuclear leukocytes, lymphocytes, and encapsulated cryptococci (×88, H&E). (C) Inflammatory cells are conspicuously absent at the intracerebral wall of the lesion with the exception of a few scattered perivascular lymphocytes (hollow arrow) (×88, H&E). (D) Within the cyst, clusters of cryptococci (solid arrow) are separated by abundant capsular polysaccharide (×220, PAS).

present within the majority of such lesions had been phagocytized by either macrophages or giant cells. In addition, PAS-stained sections contained scattered single organisms lying free within the hepatic sinusoids, apparently unphagocytized.

All lungs contained a few foci of acute pneumonitis which consisted of a sparse interstitial infiltrate of neutrophils and monocytes and dilated alveolar capillaries engorged with erythrocytes. An occasional alveolus was filled with an amorphous, eosinophilic material in the absence of an intraalveolar cellular filtrate. PAS-stained sections of the lungs contained *C. neoformans* in only two mice examined, however.

No focal inflammatory lesions were present within any spleen or kidney sections. One spleen, which was approximately twice as large as the other spleens, contained diffuse infiltrate of neutrophils within the sinusoids. Individual, apparently nonencapsulated yeast were found within the sinusoids of the PAS-stained sections of that spleen. No organisms were observed in similarly stained sections of the remaining spleens, however. No organisms were observed in kidney stained with PAS.

Demonstration of delayed hypersensitivity in immunized mice. Since mice developed protective responses at both 3 and 6 weeks after the cutaneous inoculation of viable cryptococci, it was decided that those intervals would be appropriate for the initial studies of cell-mediated immune responses, using as antigens the membrane extract (B-HEX) and SCS. Both extracts were predominantly protein (Fig. 1). Mice were footpad tested in groups of eight infected and five controls (Fig. 11). The footpad response in immunized mice peaked at week 3 and was still strong but somewhat less responsive at week 6. Both extracts were equally effective at detecting

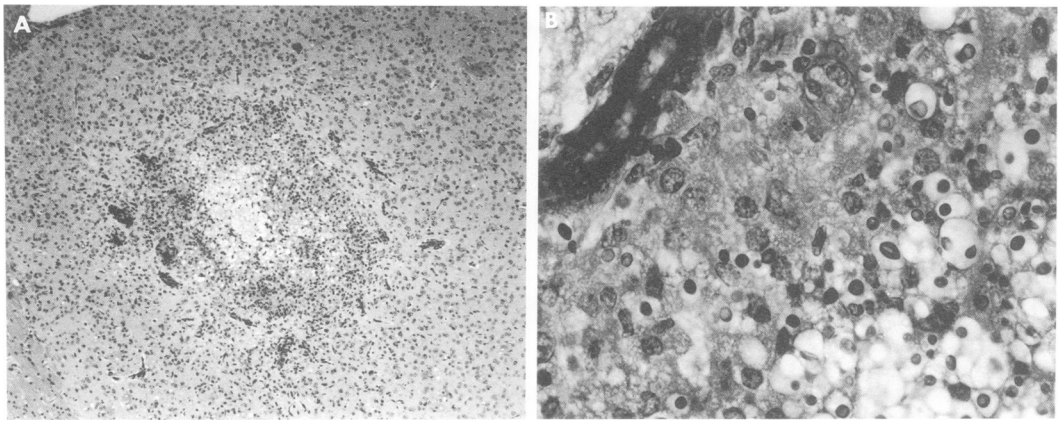

FIG. 9. (A) Focus of granulomatous cerebritis in an immunized mouse 6 weeks after intravenous challenge contains marked cellular inflammatory response comprised of macrophages, mononuclear cells, lymphocytes, and reactive gliosis (×63, H&E). (B) Yeasts are both extracellular and intracellular (×352, PAS).

the response and, although there was a large immediate response, the 24-h responses in immunized mice were greater than the 7-h responses in the same groups. In this particular experiment, the B-HEX elicited responses which were large in control mice at the early observation periods; however, that was unusual, since in later experiments responses in unimmunized mice were more like those shown for week 3. Moreover, a statistical evaluation of the data showed that the footpad responses of immunized mice to B-HEX at 3 and 6 weeks and to SCS at 3 weeks were highly significant; P values were <0.001 for all, whereas those for the responses to SCS at week 6 were significant at a P value of <0.01. There were no significant differences between the responses of immunized mice to either antigen.

Assessment of protection by culture. In an attempt to determine in a more specific and quantitative manner the protective effect of cutaneous immunization, mice which had been inoculated cutaneously with 10^4 viable cryptococci 3 weeks previously, along with previously uninoculated controls, were challenged intravenously with 10^2 cryptococci, and various organs were cultured quantitatively immediately after the intravenous challenge and then weekly for 12 weeks (Fig. 12). Data were obtainable for the unimmunized control group only through 6 weeks, as there were no survivors beyond that time. Immunized mice appeared to be able to eliminate more organisms in the first few hours after intravenous challenge, as evidenced by lower numbers in all organs, but both unimmunized and immunized animals were able to elimi-

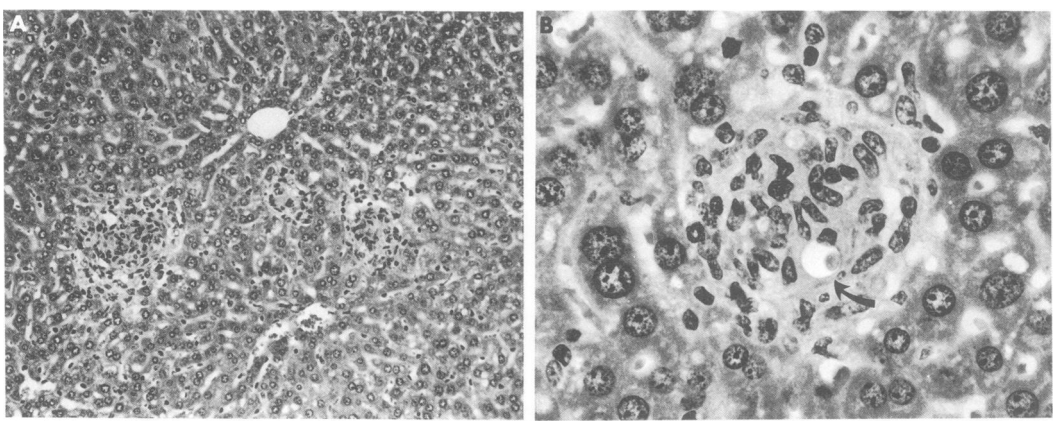

FIG. 10. (A) Liver from an immunized mouse 3 weeks after intravenous challenge shows multiple granulomas (×88, H&E). (B) A single encapsulated yeast (arrow) is visible within one of the hepatic granulomas (×352, H&E).

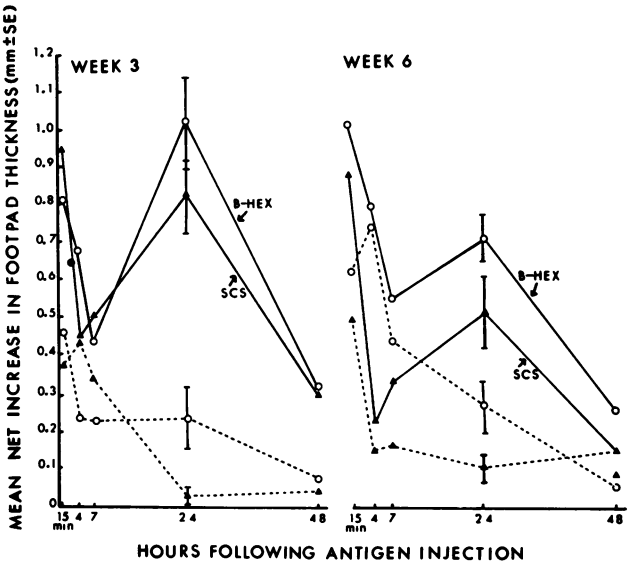

FIG. 11. Development of delayed-type hypersensitivity to a membrane extract (B-HEX) or SCS derived from the same strain of *C. neoformans*, in both control mice (---) (n = 15) and mice intracutaneously inoculated with 10^4 viable *C. neoformans* (—) 3 or 6 weeks before delayed-type hypersensitivity testing, (n = 15).

nate most organisms from the spleen, kidney, and lungs by 1 week. The liver maintained its cryptococcal load, however, as did the brain in immunized animals. Beginning at the 2nd week, the numbers of cryptococci in all organs of unimmunized mice began rising logarithmically. Moreover, there was a concomitant increase in the numbers of recoverable cryptococci from immunized mice with the exception of liver and brain, which remained stable for a few weeks; however, after the 4th week for most tissues and the 5th week for brain, the recoverable cryptococci from immunized mice were greatly reduced.

When the cultural data presented in Fig. 12 were analyzed statistically, significant differences ($P < 0.05$) between control and immunized mice were observed for all tissues at day 1. Thereafter, with the exception of the week 1 observation, the liver showed the most consistent statistically significant differences on a weekly basis between control and immunized animals. The CFU in immunized brains were significantly different from those in unimmunized brains at day 1, but significant differences were not noted again until week 4. In fact, the CFU for every organ examined at day 1 were significantly lower in immunized mice than unimmunized mice, but between weeks 1 and 4 there were no consistent patterns in the other organs.

Duration of immunity. Mice were immunized by the cutaneous inoculation of 10^4 viable cryptococci; at selected weekly intervals thereafter beginning at 2 weeks and ending at 12 weeks, groups of eight immunized and eight unimmunized mice were challenged intravenously with 10^2 cryptococci. Protection was assessed by culturing brains and livers 4 weeks after the intravenous challenge. Counts in the brain were generally higher than those in the liver, but the same relationships between unimmunized and immunized mice were observed, and therefore the data for the brains only are summarized in Fig. 13. The CFU data presented were calculated on the basis of the entire group size in the following manner. At the end of the experiment, the highest colony count from any animal was used as a normalization factor, and that factor was assigned to each mouse that had died before the appointed time of sacrifice. Where there were no survivors in a group, the normalization factor was plotted on the graph, so that those bars without standard error markings represent such groups. Protection could be detected over the entire observation period. No statistically significant difference could be demonstrated in the week 2 animals, however, but at all other weeks the data were statistically significant at the $P < 0.05$ level. Similar data were obtained for the liver cultures.

DISCUSSION

The cutaneous inoculation of viable virulent *C. neoformans* resulted in a lesion confined to the dermis with little or no dissemination of cryptococci to internal organs. Such inoculations had an immunizing effect as well, in that

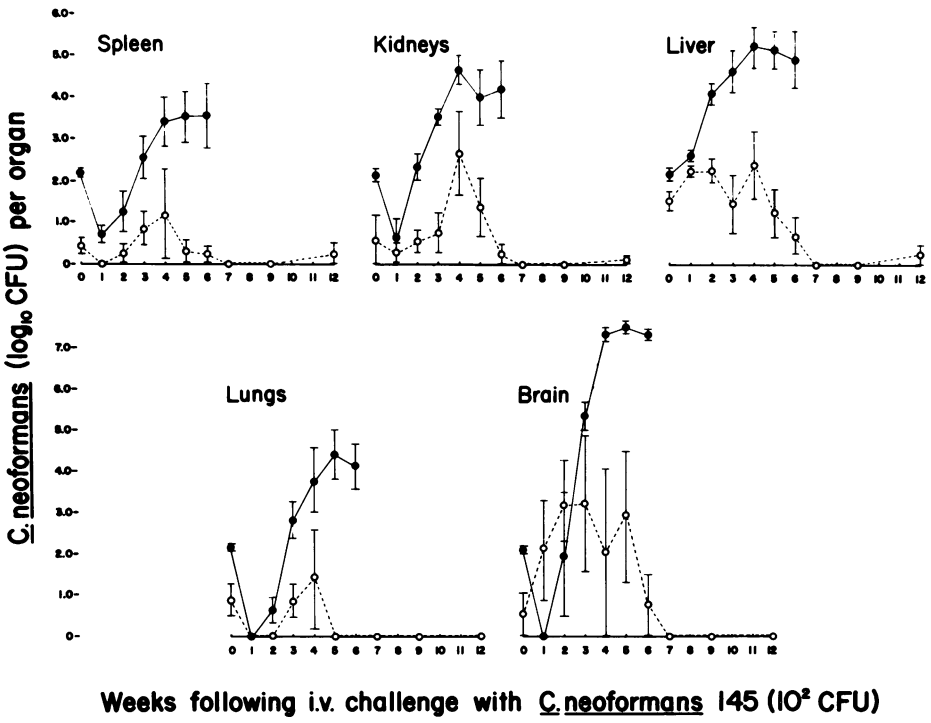

FIG. 12. Quantitative organ cultures are plotted for randomly sacrificed groups of mice (four animals per group) after intravenous challenge with 10^2 *C. neoformans*. Both unimmunized mice (—) and mice immunized by intracutaneous inoculation of 10^4 viable *C. neoformans* before intravenous challenge (---) were sacrificed 1 day postchallenge and weekly thereafter.

the protection assessed by survival or culture of brain or other organs could be detected after intravenous challenge 2 to 12 weeks after cutaneous inoculation. The cutaneous infection also resulted in the development of delayed hypersensitivity demonstrable by footpad testing with a membrane extract (B-HEX) or SCS, both of which were equally effective. The cutaneous route for the delivery of the immunizing inoculum appeared superior to intravenous (15), pulmonary (23), intraperitoneal (5, 23), or subcutaneous (8) routes, in that in each of the latter considerable systemic spread occurred. Since we wanted to use resistance to reinfection as a measure of protective immunity, it was important that the immunizing infection not disseminate. Intravenous challenge of an animal with background counts in various organs would make interpretation of the data difficult. Although we feel that the cutaneous route for immunizing with a viable inoculum is better than the other routes mentioned, it does have the disadvantage that in a certain percentage of the animals, viable cryptococci persisted at the site as long as 12 weeks. This persistent infection could serve as a continuing source of capsular polysaccharide with the potential of influencing the immune response of the animal to the challenge dose administered intravenously (20, 34).

The ability of mice immunized cutaneously to survive a systemic challenge was demonstrated clearly by both survival and culture, although the latter was a better quantitative indicator of protection. A significant indicator of the fate of the animals after intravenous challenge appeared to be their ability to eliminate viable *C. neoformans* from the brain. Graybill and Taylor (17), however, reported that they were unable to use brain CFU data to demonstrate protection. Their difficulty may have been in the fact that they cultured only at 3 and 14 days. We found significant differences in colony counts in day 1, but then not again until day 28. As has been the case in other systems, protection was fairly weak and appeared to be easily overcome. Delayed hypersensitivity was demonstrable at two selected intervals when protection was demonstrable as well, but the two events may simply have been coincident but unrelated. Dykstra and Friedman (8), using the subcutaneous route for immunization, could not detect delayed hypersensitivity but were able to demonstrate protection.

Several attempts have been made to prepare

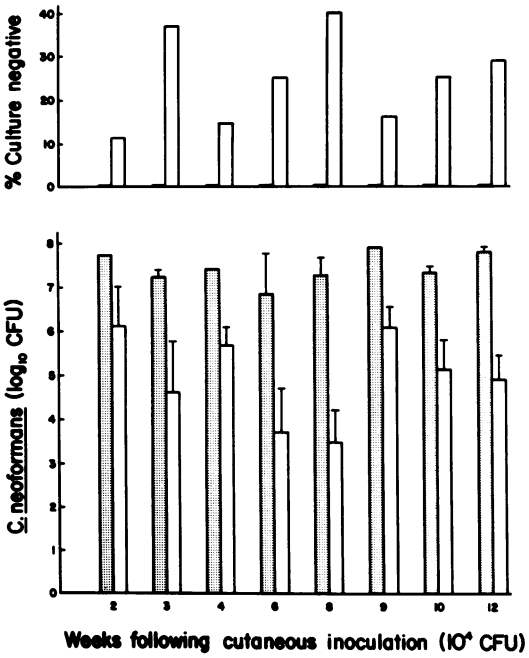

FIG. 13. Quantitative brain cultures from both unimmunized mice (stippled bars) and mice immunized by intracutaneous inoculation with 10^4 viable *C. neoformans* (open bars) 2 to 12 weeks before an intravenous challenge with 10^2 viable *C. neoformans*.

antigens suitable for detecting cellular immune responses in cryptococcus (3, 17, 19, 33, 37). Preparations tested ranged from "cell wall" extracts (37), to urea extracts (3), to culture filtrates (17, 33) and various subcellular fractions (17, 19). Dykstra and Friedman (8), however, were unable to detect delayed hypersensitivity in mice sensitized subcutaneously with viable *C. neoformans* with either the urea extract or culture filtrate antigens, and we were unable to extract culture filtrate antigens with our strain of *C. neoformans* because it produced too much capsular polysaccharide even under growth conditions which limited capsule formation. Each group tends to use its own antigen, however, and there have been few comparative studies. Those antigen preparations most like ours would be preparations used by Hay and Reiss (19) and Graybill and Taylor (17). Both preparations, however, would have contained ribosomes, whereas our cytoplasmic preparation was free of them. Furthermore, Hay and Reiss (19) had to use 125 μg of protein per dose to demonstrate the response, and their 4-h responses were always considerably larger than their 24-h responses, making the interpretation of the data somewhat difficult. Our cytoplasmic antigen was used as 20 μg of protein per dose, and the 24-h responses were greater than the 4- and 7-h responses. More detailed studies on the antigen and its use in vivo and in vitro are in progress and will be reported at a later time.

There have been several studies in which the focus of the investigation was histology of cryptococcosis or which included histology as one component of the work (4, 10, 18, 21, 32, 39). The studies of Baker and Haugen (4) and Müller et al. (32) were limited to human tissue, and the lesions observed by us in the mice were similar to those observed in humans. In most cases where dissemination was followed by histology, the animals had been inoculated intraperitoneally, but there were two instances where subcutaneous sites of inoculation were examined. Our observations of the cutaneous site are consistent with those of Song (39), who inoculated cryptococci subcutaneously, but we did not note frank abscess formation as reported by Levine et al. (21). Fazekas and Schwarz (10) found severe lung involvement after intraperitoneal inoculation, but our findings were more consistent with those of Grosse et al. (18), who also used the intraperitoneal route for inoculation, in that we found only minimal lung pathology. We and others (10, 18) found spleens and kidneys surprisingly free of histopathology. The lack of histopathology in spleens and kidney and the minimal pathology in lungs was surprising in view of the CFU obtained from those organs. Tissue reaction, or lack thereof, in the brain as demonstrated by us and others (10, 18, 21) was highly variable, including instances of cyst formation, granuloma formation, or free cryptococci with essentially no host response. In evaluating the overall tissue responses observed by us in these animals, however, two features were obvious. First, surprisingly, there were no observable differences in tissue response between immunized and nonimmunized animals; second, in the majority of the lesions observed the predominant cell was the macrophage mixed with a few polymorphonuclear leukocytes and lymphocytes.

In summary, we have characterized an experimental model of cryptococcosis in which the primary immunizing infection is self-contained and in which delayed hypersensitivity and protection can be measured. This should be a good model in which to study immunoregulatory aspects of cryptococcosis as well as other host-parasite interactions.

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