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Pathogenesis, Lethality, and Immunizing Effect of Experimental Cutaneous Cryptococcosis

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Mice were subcutaneously inoculated with small numbers of virulent *Cryptococcus neoformans* and divided into groups. Numbers of viable yeasts at the site were estimated at weekly intervals for 5 weeks on the basis of cultures of minced tissue excised from sacrificed animals. Organisms multiplied at the site for at least 4 weeks and were still detectable after the 5th week, although in reduced numbers. Agglutinins appeared within a week, but these antibodies were not detectable during the 2nd through the 5th week. Cryptococcal polysaccharide began to appear in the sera at 3 weeks, persisting through the duration of 5 weeks. All animals appeared healthy, but a few sickened after many months and died of systemic cryptococcosis. All of these events were observed in many separate experiments. The immunizing capacity of a cutaneous lesion was tested by challenging some of the above animals with viable *C. neoformans* after various intervals of time, either subcutaneously at a site distant from that of the vaccination or intravenously. Although we were unable to demonstrate reduced multiplication of yeasts in the brains, lungs, and spleens of intravenously challenged animals, it was possible to show that multiplication was inhibited at the site of subcutaneous challenge. It was noted also that vaccinated animals lived longer after lethal intravenous challenge than did nonvaccinated animals. The latter protection was observed, however, only when challenge followed vaccination by 3 weeks or longer, and it was effective only against a relatively low challenge dose. Mice were protected against a higher dose if they had previously received killed cryptococci, alternating subcutaneous and intraperitoneal inoculations, one of which contained a microbial adjuvant. No protection was observed in animals that were subcutaneously vaccinated with inert materials such as chitin, latex spheres, or even cryptococcal cell walls themselves.

Systemic cryptococcosis is thought usually to be acquired by inhalation of infectious particles of *Cryptococcus neoformans*. It is unknown whether naturally acquired systemic disease is ever a consequence of subcutaneous implantation of viable organisms. Cutaneous cryptococcosis has been produced experimentally in a variety of animals, but usually these infections resolved spontaneously (4, 11, 15, 21, 22, 24). The exceptions were attributed to inadvertent inoculation into deeper tissues (e.g., 15).

It is also unknown whether a primary dermal lesion can have an immunizing effect upon a host. While our own investigations were in progress, Staib and Mishra (23) reported that the incidence of deaths among mice inoculated intramuscularly with a highly virulent isolate was significantly reduced when the animals previously had received an intramuscular injection of a different, avirulent isolate. Their results are not directly applicable, however, to events in naturally acquired infection, since primary intramuscular cryptococcosis has not been reported to our knowledge, and the chance of such occurrence would be minimal compared with implantation into the skin. Primary cutaneous lesions have been observed, albeit rarely (20), and might even be a not infrequent although unrecognized occurrence, a presumption based upon the widespread distribution of this yeast in nature.

Experiments are described herein in which mice were subcutaneously inoculated with relatively small numbers of virulent *C. neoformans* and observed for morbidity and mortality. Companion animals so vaccinated were challenged by different routes after various time intervals to determine the immunizing capacity of an initial subcutaneous lesion. Morbidity and mortality, histological response, serologies, and tests for delayed hypersensitivity were compared between vaccinated and nonvaccinated animals. In parallel with some of these experiments, mice were inoculated with substances known or suspected to incite an inflammatory response,

namely, chitin, latex particles, and cryptococcal cell walls. Other mice were subcutaneously and intraperitoneally vaccinated with killed cryptococci plus *Bordetella pertussis* adjuvant, a method first used by Abrahams in 1966 (1). These animals also were challenged and observed for morbidity and mortality. It is intended that these studies provide a base for investigating host defense against this disease.

MATERIALS AND METHODS

Experimental animals and preparation of yeast inocula. *C. neoformans* 145, originally isolated from human spinal fluid, was used throughout. Though heavily encapsulated on ordinary agar media, encapsulation was suppressed for these experiments by growing the organisms on Sabouraud agar or broth, each containing 16% glucose at pH 5.0 (5). When viable cells were to be inoculated into animals, the cells were harvested from agar plates and suspended in sterile phosphate-buffered saline (PBS), and the concentration was adjusted such that a desired dose could be administered in a particular volume: 0.5 ml for inoculation of subcutaneous tissue at the back of the neck above the shoulder blades; 0.5 or 0.1 ml subcutaneously into a shaved right flank; or 0.5 ml intravenously into a lateral tail vein. The concentration of organisms in the inocula was confirmed by means of replicate pour plates prepared at the time of the inoculations.

Cell walls were prepared from organisms grown in broth for 72 h at 37°C, agitating at 150 gyrations/min, and then harvested by centrifugation at 10,000 × *g* for 30 min. Packed cells were washed three times by resuspension in PBS followed by centrifugation as before. After the final wash, the cells were suspended in a volume of PBS equal to that of the packed cells and killed by holding the suspension at 70°C for 1 h. Sterility was confirmed by streaking the suspension onto Sabouraud agar plates. Two drops of Tween 80 were added to 20-ml samples of the killed-cell suspension, and then each sample was agitated violently with 20 g of 0.25- to 0.30-mm sterile glass beads in a Braun homogenizer (Bronwill Scientific, Rochester, N.Y.). At 1-min intervals during homogenization, lactophenol cotton blue wet mounts were examined microscopically. Eighty to 90% of the cells had been broken by 10 min, at which time homogenization was discontinued and the glass beads were allowed to settle (about 5 min). The homogenate was removed and centrifuged at 1,000 × *g* for 15 min. This pellet was discarded, and the supernatant fluid was centrifuged at 12,000 × *g* for 30 min. After six washes, the final pellet was suspended in sterile PBS, the volume of which was nine times that of the pellet. Microscopic examination revealed that most, though not all, of the whole cells had been removed, leaving mostly ruptured cells and cell wall fragments. Despite the violence of the homogenization, however, all cell wall fragments seen had capsular material that adhered to the external portion of the wall as noted in India ink preparations.

Cryptococci used for killed-cell vaccines were collected from the "high-glucose" Sabouraud agar plates into sterile PBS containing 0.5% formalin and kept at ambient temperature for 2 days before testing for sterility. The cells were washed three times in sterile PBS, adjusted to contain 8×10^8 organisms per ml on the basis of triplicate hemocytometer counts, and stored at 5°C.

Latex particles (5.7 μm) were purchased from Dow Diagnostics. Crustacean chitin (Calbiochem) that had been powdered in a mortar and pestle was contributed by Judith Domer. Each was suspended in sterile PBS at a concentration resulting in a volume of packed solids that was 10% of the total volume when centrifuged for 15 min at 1,000 × *g*.

Male CD-1 mice (Charles Rivers), 4 to 6 weeks old, were used throughout.

Determination of yeast population and histology in host tissues. Viable yeasts at the sites of subcutaneous inoculations were estimated by sacrificing animals and culturing the minced subcutaneous dermis on ordinary Sabouraud agar plates containing 0.005% chloramphenicol, three plates per site. The skin was removed and the inner surface was smeared over one plate. The remainder, which was subcutaneous tissue, was divided into two portions, each of which was minced, and the mincings were smeared over plates. Cryptococcal census in organs was determined by aseptic removal and homogenization of the organ in 2 ml of sterile PBS, diluting the homogenates in 10-fold increments, spreading 0.2 ml of each dilution onto each of three agar plates, and counting the colonies after an appropriate period of incubation.

Histological responses were evaluated by examining sections stained with hematoxylin-eosin and with periodic acid-Schiff or mucicarmine stain.

Tests for delayed hypersensitivity and serologies. Mice were tested for delayed hypersensitivity with either a 1:10 or a 1:5 dilution of Bennett-type (2) or undiluted Murphy-type (18) cryptococcin prepared in this laboratory. Both were of comparable potency in sensitive guinea pigs to material kindly supplied by John Bennett. For the experimental groups, 0.03 or 0.02 ml of skin test antigen was inoculated into the left hind footpad or into the right external ear flap, and thickness of footpad and ears was measured by calipers (Schnelltaster, H. C. Kroplen, GmbH, Schluchtern, West Germany) at 0.5, 4, 24, 48, 72 h. Initial thickness, measured just before administration of the test material, was subtracted from the reading, and the difference, if any, was considered as increase in thickness.

Sera of mice were tested for anticryptococcal agglutinins according to the method of Gordon and Lapa (9), using charcoal particles (kindly provided by Joseph Portnoy of Hynson, Wescott, and Dunning, Inc.) coated in our laboratory with cryptococcal polysaccharide from the same isolate used for the other experiments. Latex particles coated with rabbit anticryptococcal polysaccharide antibodies (International Biological Laboratories, Rockville, Md.) were used to detect cryptococcal polysaccharide (4); control materials were provided by the manufacturers with the kit. (These particles will detect at least 0.025 to 0.030 μg of polysaccharide [personal communication from the manufacturer]. According to Kaufman and Blumer [12], the latex test is 100% specific and 95% sensitive with clinical specimens.) Serum containing cryptococcal antibody was kindly supplied by Morris Gordon. Negative sera were obtained from noninfected mice.

RESULTS

Pathogenesis of cutaneous infection. Thirty-five mice were inoculated subcutaneously into the back of the neck with 10^2 viable units of cryptococci, and 10 animals were saved. The remainder were exsanguinated (five each week for 5 weeks), the sera were saved, and the inoculation site was excised. Organisms were recovered from these sites in increasing numbers for four weeks, but by the 5th week the numbers had begun to diminish. Two of the animals that had not been sacrificed eventually died of systemic cryptococcosis; they appeared entirely normal, however, until about a week before death, 70 to 80 days after inoculation. At autopsy there were grossly discernible lesions on the brain, lungs, spleen, liver, and kidneys, and these lesions contained encapsulated yeasts. Cultures of these organs grew numerous cryptococcal colonies. The other eight appeared healthy during 118 days of observation.

Significant titers of anticryptococcal agglutinins were demonstrable in the sera of mice sacrificed at the end of the 1st week, but not in those sacrificed during the remainder of the 5-week period. Sera from noninfected animals were consistently negative. Cryptococcal polysaccharide, on the other hand, usually was detected in the sera, beginning with the 3rd week after inoculation. Estimates of numbers of cryptococci in subcutaneous tissue, antibody titers, and presence of cryptococcal polysaccharide antigen are shown in Table 1.

TABLE 1. *Course of events during pathogenesis of subcutaneous cryptococcosis induced in nonvaccinated mice: appearance of cryptococcal polysaccharide and disappearance of antibody, correlated with multiplication of organisms*

Time after inoculation[a] (weeks)	Mouse no.	Yeast cultured from site[b]	Agglutinins[c]	Cryptococcal polysaccharide in serum
1	1	++	1:8	–
	2	++	1:8	–
	3	+	–	–
	4	+	1:4	–
	5	None	1:4	–
2	6	+	–	–
	7	+++	–	–
	8	++	–	–
	9	+	–	–
	10	+	–	–
3	11	+++	–	+
	12	+++	–	–
	13	None	–	–
	14	+++	–	+
	15	+++	–	–
4	16	+++	–	+
	17	++++	–	–
	18	++++	–	+
	19	+++	–	–
	20	+++	–	+
5	21	++	–	+
	22	++	–	+
	23	++	–	+
	24	+++	–	+
	25	+	–	+

[a] Mice were inoculated subcutaneously with 10^2 viable units of *Cryptococcus neoformans.* Periodically, groups of mice were killed, and the sites of inoculation were excised, minced, and smeared over agar plates, three per site.

[b] +, Occasional colonies; ++, less than half the plate covered with growth; +++, more than half the plate covered with growth; ++++, confluent growth.

[c] Against charcoal particles adsorbed with homologous polysaccharide.

Immunizing effect of cutaneous infection (intravenous challenge). During the first of a series of experiments, 20 mice were vaccinated by inoculating 10^2 viable units subcutaneously. Twenty-one days later, these mice, as well as 20 control mice that had not been vaccinated, were intravenously challenged with 10^2 viable units of *C. neoformans.* Vaccinated mice died at a slower rate than did the control mice, although deaths began to occur in both groups at about the same time (Fig. 1).

A second experiment was done in which 53 mice were vaccinated subcutaneously and challenged intravenously along with 53 control mice precisely as before, except that the mice were challenged sooner, at 7 rather than 21 days. Twenty of each of the vaccinated and control groups were held for observation, and 33 of each were set aside to determine the in vivo fate of organisms. Of those held for observation, no protection was observed; during a period of 65 days, there was no difference in daily cumulative mortality (Fig. 2).

Of those saved for investigating the fate of the organisms, three vaccinated and three control animals were sacrificed immediately after challenge and at 1 through 8, 11, and 18 days. The brains, spleens, and lungs were cultured quantitatively. Colony counts on replicate plates of a single organ homogenate did not vary to any extent at any time, but agreement of counts within any one group of three animals depended upon the time after inoculation that the three were sacrificed. Immediately after inoculation, cryptococcal populations of the mice were essentially the same, and, in fact, much of the inoculum was accounted for in the lungs. After 24 h, there was still good agreement within each group of three, and there was no difference between vaccinated and control animals, although there was a decided drop in yeast counts of the lung homogenates of both groups, which could not be explained by dispersion to either of the other two organs. By 48 h, however, the yeast census

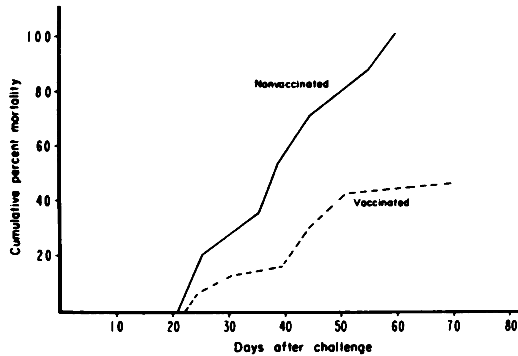

FIG. 1. *Protection of mice by subcutaneous vaccination with 10^2 viable units of C. neoformans 3 weeks before intravenous challenge with 10^2 viable units of C. neoformans, 20 animals per group.*

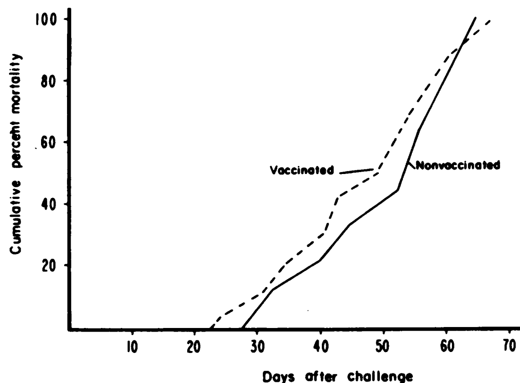

FIG. 2. *Failure to demonstrate protection among mice challenged intravenously with 10^2 viable units of C. neoformans, 1 week after subcutaneous vaccination with the same number of viable units of the same isolate, 20 animals per group.*

began to vary widely even within a single group of three animals of the same experimental background, varying from no organisms to 20 times the concentration of the challenge inocula. When there had been multiplication, as in the latter case, the organisms were sometimes in greatest numbers in the brain, but at other times in the lungs. This variability of yeast census persisted throughout the remainder of the days. Significant differences between vaccinated and nonvaccinated animals in terms of in vivo multiplication of the organisms was not detectable, even by averaging the yeast censuses.

In a third experiment, we decided to again use an interim of 21 days between vaccination and intravenous challenge, since results of the second experiment indicated that challenge after only 7 days may have been too early. But we decided also to include an additional group, to be given a higher challenge dose, because we thought that the small number of cells used for challenge in the second experiment had possibly been a factor in the wide variation in numbers of yeasts detected in the organs, reflecting a possibly greater variability of host-parasite relationships with a low challenge dose. Seventy-three mice were vaccinated. Twenty were challenged with 10^2, as previously, and 53 were challenged with 10^3 organisms. Of those receiving 10^3, 33 were sacrificed as before, their organs were cultured quantitatively, and the counts were compared with those of equal numbers of nonvaccinated control animals. As in the previous experiment, the numbers of yeasts in the organs varied greatly, and significant differences between control and vaccinated mice could not be distinguished. The remaining group of 20 vaccinated animals challenged with 10^3 and the 20 given 10^2 were observed for 70 days, in parallel with equal numbers of nonvaccinated controls, and the cumulative deaths were recorded. There was again evidence of a degree of protection among the vaccinated mice challenged with the lower dose; as in the first experiment, vaccinated mice lived longer than controls (Fig. 3). A similar protection was not seen among mice challenged with the dose of 10^3.

Protection was confirmed in yet a fourth experiment in which vaccinated mice in two groups of 20 were challenged 21 days later with 10^2 or 10^3 viable units intravenously, and their death curves were compared with those of nonvaccinated control mice concurrently infected. Protection was again observable, but only when the lower challenge dose was used (Fig. 4).

The effect of length of time between vaccination and challenge and its relationship to protection was further examined in a fifth experiment. One hundred and twenty mice were subcutaneously vaccinated with viable organisms as pre-

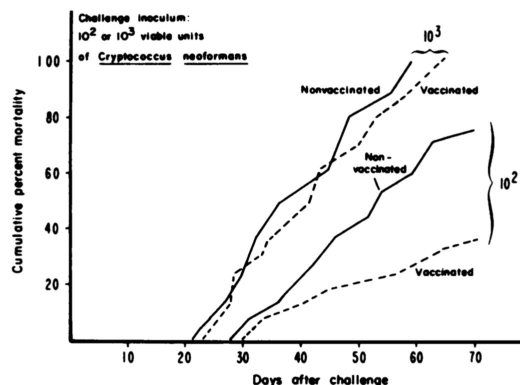

FIG. 3. *Effect of challenge dose upon demonstration of protection among mice subcutaneously vaccinated with 10^2 viable units of C. neoformans 3 weeks before intravenous challenge, 20 animals per group.*

viously. At the end of each week for 6 weeks, 20 vaccinated and 20 control mice were challenged intravenously with 10^2 viable units of cryptococci. There was no detectable protection when challenge followed vaccination by only 7 days (Fig. 5). It was observed, however, that animals evidenced varying degrees of protection when challenge was delayed until 3 to 6 weeks after vaccination, and the strongest protection occurred in those animals challenged at the longest interval.

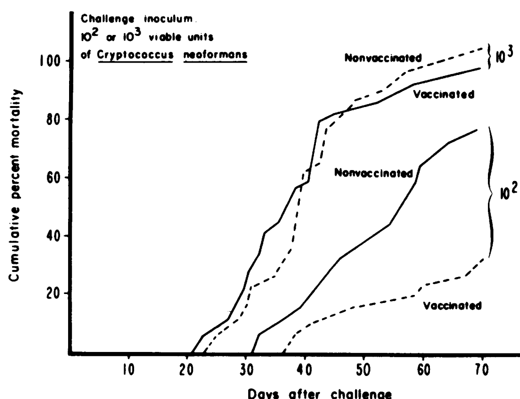

FIG. 4. *Confirmation of the effect of challenge dose upon demonstration of protection among mice subcutaneously vaccinated with 10^2 viable units of C. neoformans 3 weeks before intravenous challenge, 20 animals per group.*

Immunizing effect of cutaneous infection (subcutaneous challenge). Sixty-five mice vaccinated with viable organisms subcutaneously in the neck and 65 nonvaccinated controls were challenged by inoculating 10^3 viable yeasts particles suspended in 0.5 ml of PBS into the subcutaneous tissue of the right flank 3 weeks after vaccination. Twenty of each group were held for observation, 30 of each were held for histological studies, and 15 from each were used to monitor survival of the challenge inoculum. For the latter, five mice from each group were sacrificed at 1, 3, and 7 days after challenge, the sera were saved, and the sites of subcutaneous challenge were excised and cultured. The organisms disappeared from the site in vaccinated mice; organisms were recovered after 1 and 3, but not 7, days, whereas control mice had viable yeasts at the challenge site at each observation.

FIG. 5. *Relationship of protection to interval of time between subcutaneous vaccination with 10^2 viable units of C. neoformans and intravenous challenge with 10^2 units of the same isolate, 20 animals per group.*

These data were confirmed in an experiment in which 50 vaccinated and 50 control mice were challenged as before except that the yeasts were suspended in only 0.1 ml of PBS. One, 3, 5, 7, and 14 days after challenge, the inoculation site of 10 mice from each group was excised and cultured. The numbers of colony-forming units culturable from the flanks of the nonvaccinated mice increased significantly throughout the observation period, whereas the numbers from vaccinated animals did not.

Of the 20 vaccinated and nonvaccinated animals that were challenged and saved for observation of morbidity and mortality, 18 of each group remained grossly normal through 100 days of observation, but 2 of each died at 50 and 69 days, respectively, of systemic cryptococcosis with extensive cerebral involvement. Until a few days before death, these four mice also had appeared normal, and, in fact, no balance disorders were discernible that would have indicated meningeal involvement.

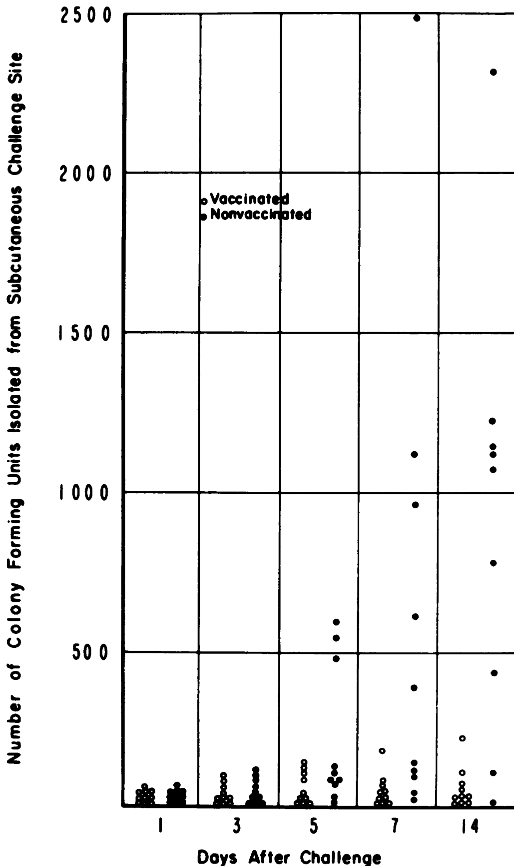

FIG. 6. *Inhibited multiplication of C. neoformans in subcutaneous tissues of vaccinated mice. Mice were vaccinated with 10^2 viable units of C. neoformans inoculated into the subcutaneous tissue of the neck and challenged 3 weeks later with 10^3 viable units of the same isolate inoculated subcutaneously into the right flank. Each point represents one animal.*

The animals set aside to study histological reaction were sacrificed in groups of five vaccinated and five nonvaccinated at 1 h and 1, 2, 3, 5, and 7 days after challenge, and the sera were saved. Histopathological sections of the tissues at the challenge sites revealed no apparent difference in response between the two groups of mice; both showed a transient mononuclear infiltrate that disappeared by the 5th day. Interpretations of the histology were confirmed by Roger D. Baker.

The sera obtained when mice were sacrificed for the histological study or the yeast survival were tested for agglutinins and cryptococcal polysaccharide antigen. Agglutinins were demonstrable in the nonvaccinated control mice, but on only 1 day—the 7th after challenge—confirming the transient appearance of anticryptococcal antibodies that was noted previously. It was observed also that vaccinated mice had serum levels of cryptococcal polysaccharide at the time of challenge, although the polysaccharide could not be detected 2, 3, and 5 days after challenge. By the 7th day, polysaccharide could again be demonstrated. In nonvaccinated mice, polysaccharide could not be demonstrated in sera at any time during the 7 days of observation after inoculation.

Immunizing effect of killed cryptococci administered with a microbial adjuvant. Sixty-two mice were vaccinated according to the procedures of Abrahams (1), which consisted of a series of inoculations with killed cryptococci, alternating between intraperitoneal and subcutaneous routes, and with *B. pertussis* added to the next to the last inoculum. Control groups consisted of: (i) 62 mice given no vaccine of any kind, (ii) 20 mice receiving only bacteria, and (iii) 20 mice given only killed yeast. All four groups were challenged intravenously with 10^3 viable units of *C. neoformans* on the 7th day after the last inoculation of vaccine into the experimental group.

Twenty mice from each of the groups were observed for morbidity and mortality. The mice vaccinated with yeasts combined with the adjuvant were more effectively immunized than any of the other groups, as manifested by longer survival (Fig. 7). Mice vaccinated with cryptococci without adjuvant seemed also to have extended survival rates, although not to the same extent as when adjuvant was used. Animals receiving only adjuvant died at the same rate as nonvaccinated animals.

Forty-two of the experimental mice and 42

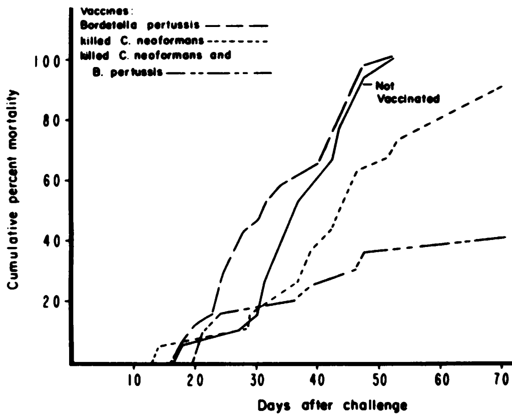

FIG. 7. *Survival of variously vaccinated mice when challenged 21 days later with 10^3 viable units of C. neoformans intravenously, 20 animals per group.*

from the control group given no vaccine were used to monitor in vivo yeast multiplication, presence of antibody or antigen, and histological responses. At 1, 6, 12, 18, and 24 h and at 2 through 8, 11, and 18 days, three animals from each of the two groups were exsanguinated and the sera were saved. The left lung lobes were removed for determination of yeast counts. The numbers of yeasts isolated followed the same pattern of variation as in previous experiments with intravenously challenged animals, and vaccinated animals could not be distinguished from the controls on this basis. Organisms could not be seen in sections of the lungs until the infection was rather advanced (about day 6). At this time, the lungs of both the vaccinated and control animals had an intense inflammatory infiltrate around heavily encapsulated yeasts. Antibody was not detectable at any time throughout this experiment in either the vaccinated or control animals. Cryptococcal polysaccharide, however, was present in the sera of vaccinated animals by the day of challenge (i.e., 21 days after the first vaccination) and every day thereafter. In control mice, polysaccharide antigen began to appear when the disease became extensive, about 18 days.

Effect of chitin, latex particle, and cell wall inocula (intravenous challenge). Groups of 25 mice were inoculated subcutaneously with 0.1 ml of 10% (vol/vol) suspensions of: (i) powdered chitin, (ii) latex spheres, and (iii) cell wall fragments. A fourth group of 25 mice received 0.1 ml containing 10^2 viable units of *C. neoformans.* Seven days later, the animals from the groups that had received any of the inert materials had palpable nodules at the sites, whereas the animals vaccinated with live cells did not.

Five mice from each group were sacrificed, and the excised inoculation site was fixed in Formalin. Stained sections revealed large amounts of each inert material surrounded by an intense inflammatory response, characterized by infiltration of both polymorphonuclear leukocytes and mononuclear cells. The live-cell vaccine, on the other hand, apparently elicited no inflammatory response; however, cryptococci could be seen in only one tissue specimen out of five.

The surviving animals of the original four groups as well as 20 nonvaccinated control mice were challenged with 10^2 viable units of *C. neoformans* and observed 50 days for morbidity and mortality. Animals previously inoculated with chitin, latex particles, or cell walls died at the same rate as control mice. Mice vaccinated with live cells, however, died at a slower rate, although early death occurred at about the same time as did the first deaths in the groups in which protection was not evident.

Tests for delayed hypersensitivity. At several points during most of the previously described experiments, tests for delayed hypersensitivity were performed. Mice that had been given a single cutaneous inoculation of live cells were tested 1, 17, and 24 days after infection; those that had been vaccinated with live cells and intravenously challenged with live cells were tested 7 and 21 days after challenge; and those that had been vaccinated with killed cryptococci and microbial adjuvant were tested 8 and 21 days after challenge. Responses to the two skin test antigens were consistently negative; average thickness of the ears or footpads in both experimental and normal mice were comparable in size and always had returned to the original thickness by 24 h.

DISCUSSION

The initial purpose of these experiments was to investigate mechanisms of host defense in cryptococcosis. That there is a good defense seems obvious inasmuch as the agent is widespread in nature, and therefore most individuals must be frequently exposed even though relatively few develop the disease. Several studies have failed to demonstrate protection of animals after immunization (S. Marcus and R. F. Rambo, Proc. Soc. Am. Bacteriol. 8:92, 1955; 7, 10). In vitro experiments suggest an effective nonimmune defense by phagocytosis (16), but the net effectiveness in terms of resistance to disease within an intact host has not been demonstrated. Neither has it been possible to show stimulation of a protective immunological defense engendered by infection, although it seems plausible that the immune system is involved, inasmuch

as cryptococcal disease is seen primarily among individuals whose immune mechanisms, specifically the cell-mediated system, have been compromised. Furthermore, Abrahams (1) clearly demonstrated protection among animals given multiple injections of killed yeast and adjuvant.

We decided to first investigate the consequence of a subcutaneous infection, reasoning that the likelihood of inducing a protective response, be it immunological or not, would be as a result of an actual infection. Also, such a system would easily allow use of only a single isolate for both vaccination and challenge, as opposed to vaccination with an avirulent isolate and challenge with another, thus avoiding possible complication by heterogenous antigenic subtypes. Additionally, it was considered that subcutaneous infection might not be infrequent as a natural event in epidemiology of human disease.

This approach required knowledge of the pathogenesis of cutaneous infection. Accordingly, mice were subcutaneously inoculated with viable *C. neoformans*, and, as anticipated, subcutaneous cryptococcosis was a benign experience for the great majority of the animals. Nevertheless, such lesions did not quickly resolve. Rather, the yeast inoculum survived and multiplied for at least 4 weeks, although elimination of viable organisms did begin during the 5th week. Animals almost always remained free, however, of grossly discernible lesions such as nodules or ulcers; there were only three instances during this entire study when palpable lesions appeared at the site of a subcutaneous infection, and these were histologically granulomatous. It was additionally apparent that multiplication of organisms was usually checked at some point, since aged mice almost always appeared healthy and had neither abscesses nor any gross evidence whatsoever of infection. In a few instances, however, some of these animals died of systemic cryptococcosis after months of appearing entirely normal. This low incidence of mortality differs from the observations of Bergman, who in 1961 found that large numbers of viable cryptococci inoculated into the dorsum of a hind foot resulted in death in a majority of his mice (3). Our results are compatible, however, with most studies in that deaths were few after subcutaneous inoculation (e.g., 11, 21, 22). Although it seems reasonable to assume that subcutaneous inoculation is only infrequently followed by hematogenous or lymphatic spread, as was evident by the rarity of deaths observed by us and others, and by analogy with other fungus diseases (e.g., coccidioidomycosis and blastomycosis; 25), a recent study by Song (22) indicates that this may not be so. She cultured liver, spleen, lungs, and brain from mice that had been subcutaneously inoculated with 2×10^3 viable units of virulent *C. neoformans* 7 or 14 days previously and found viable yeasts in all, despite the fact that only a few of her animals died of systemic cryptococcosis. We do not know how many of our animals experienced systemic spread after subcutaneous inoculation; it was the purpose of our experiment to determine the eventual outcome of long-term undisturbed cutaneous cryptococcosis in significantly large groups of animals. The few deaths that eventually occurred demonstrated that systemic spread assuredly occasionally took place, and Song's data imply that such spread is the rule, even though infrequently terminating in death.

Our experiments did demonstrate that subcutaneous implantation of viable yeasts confers a degree of protection against subsequent challenge, albeit minimal; vaccinated mice lived longer after intravenous challenge and were able also to control multiplication of organisms at the site of a subcutaneous challenge. We were unable, however, to use suppressed multiplication of cryptococci as an indicator of protection in intravenously challenged animals, because within a few days after such an inoculation, yeast populations began to vary greatly within samplings of identically treated animals. The reality of the variation in counts observed 48 h and longer after challenge is fortified by the consistent numbers obtained from animals sacrificed immediately and within 24 h after challenge, indicating that the technique itself was sensitive and precise. Even cultures of animals that were negative probably were misleadingly so inasmuch as all of companion animals held for observation eventually died of cryptococcosis, suggesting sequestering in the negative, sacrificed animals. The erratic numbers of yeasts cultured from organs in both vaccinated and nonvaccinated animals might be accounted for, at least in part, by the chronic nature of cryptococcosis. Unlike what is seen with many bacterial pathogens, variations in the disease process may occur naturally among different individuals, and are to be expected especially in response to relatively avirulent inocula such as 10^2 viable colony-forming units of the isolate used in our experiments.

Demonstration of even the minimal protection that was achieved was difficult, requiring relatively large numbers of animals to obtain reproducible results, and yet other problems were encountered that had to be overcome before protection could be detected. For example, the challenge dose was critical. Subcutaneous vaccination was effective against intravenous challenge of 10^2 viable units, but resistance was overwhelmed by 10^3. Furthermore, the respon-

sible mechanism, whatever it was, had to be allowed to mature for at least 3 weeks, for protection was not observed if intravenous challenge followed an interim of less than that—indeed, protection was greatest when the interim between vaccination and challenge was longest, 5 to 6 weeks in our experiments. A problem that was not recognized until too late was our failure to determine whether the intravenously challenged vaccinated animals, which evidenced prolonged survival, were in fact able to sterilize their infections. Had this rather inexplicable oversight not occurred, better data would be available indicating more precisely the status of those animals that remained without overt signs of disease despite introduction of the infectious agent.

Was the protection we observed a manifestation of an educated immunological response? None of our data proves that it was. It is probable that our protected animals were superinfected, in that the preliminary studies on pathogenesis showed that subcutaneously inoculated animals did not begin to control their lesions until after the interval of time we chose for challenge. In fact, a number of factors point to the possibility that the protective mechanism was nonspecific, primed by the presence of a subcutaneous pocket of foreign material. The protection was minimal and incomplete; resistance was easily overwhelmed; vaccinated animals did not exhibit a delayed hypersensitivity response (a correlate of specific immune protection in most systems) to two types of cryptococcin, despite concurrent demonstration of marked activity of the materials in guinea pigs sensitized with *C. neoformans* (unpublished data); and histological preparations did not indicate any immunological mechanism that might be construed as being protective, such as flooding of the site with lymphocytes and macrophages after inoculation into vaccinated animals. We considered the possibility that the protection observed was nothing more than excitement of host inflammatory cells, even though there is no precedent in medical literature insofar as we are aware. To the contrary, Kellermeyer and Warren (13) have reported that animals respond to an injection of inert particles (plastic beads) with identical time course and cell populations irrespective of whether or not the injection has previously occurred. To test our hypothesis, nevertheless, an intense inflammation was induced in animals by inoculation of inert particles before challenge with viable cryptococci. No protection was seen. In fact, not even inoculation of cryptococcal cell walls could engender protection.

The question remains, is an immunological mechanism involved in keeping human beings free of disease? Further, what confers resistance to disease in a primary infection? The latter would seem to be the more important question if, in fact, infections are frequent, as has been presumed. The concept of frequent infections is not without its detractors, however, because of difficulty in understanding how a particle as large as *C. neoformans* can easily pass through the respiratory tract and lodge within an alveolus. Bulmer and his associates have suggested that only the nonencapsulated yeast is small enough to accomplish this feat and, further, that cryptococci in soil tend to be nonencapsulated (6). An alternate explanation for the infectiousness of *C. neoformans* can be offered based upon the recent report of Kwon-Chung (14), who has described the sexual spore of the perfect stage of this organism, the basidiospore, which by virtue of its small size (1.8 to 2.5 μm) could more easily make its way deep into the lungs, but the distribution of this form in nature cannot be speculated upon at this time. If the basidiospore should prove to be the infectious unit and is sparsely distributed in nature, then infection may be infrequent, despite preliminary skin test data suggesting that approximately a third of human beings are positive to cryptococci (17).

A final aspect of our study may have bearing upon the consensus that heavily encapsulated cryptococci are less antigenic (i.e., stimulate lower antibody levels) than are cryptococci that are small capsuled or nonencapsulated or from which the capsule has been chemically removed (10, 19). It has been our experience, as well as that of others (e.g., 12), that humans with cryptococcosis have polysaccharide rather than antibody in their serum, and that in human disease the organism usually is heavily encapsulated. It is probable that freed polysaccharide, sloughed from heavily encapsulated cells, reacts with and masks the presence of antibody. We invariably observed polysaccharide in the serum of mice after subcutaneous inoculation of viable nonencapsulated cryptococci, but shortly after administration of a second inoculation of the same kind a few weeks later, the polysaccharide disappeared, reappearing days later. It is difficult to explain this disappearance except by assuming that the minimally encapsulated cells in the second inoculation stimulated antibody that bound the surplus polysaccharide. One problem in making such an assumption arises in attempting to explain why antibody was never in excess, and thus detectable in at least some of our animals. We suspect that at times antibody was, in fact, in excess but was not detected because of a lack of sensitivity in the test for antibody, as contrasted with the exquisite sensitivity of the test for polysaccharide. In any case, if the

presumption is made—and admittedly the presumption is highly speculative—that the temporary disappearance of polysaccharide was due to binding by antibody, it can be concluded that a booster effect occurred as a result of the second inoculation. Irrespective of the mechanism, there is an important counterpart in human disease. From a diagnostic standpoint, it is important to emphasize that whereas antibody often is absent, polysaccharide often is present and is easily detected by a simple laboratory test using commercially available reagents.

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