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Response of Congenitally Athymic (Nude) and Phenotypically Normal Mice to *Cryptococcus neoformans* Infection

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A *Cryptococcus neoformans* infection in congenitally athymic (nude) mice and phenotypically normal heterozygote BALB/c mice was used to determine how T lymphocyte-deficient mice compared with normal mice in restricting proliferation of *C. neoformans* and to determine whether a correlation exists between delayed-type hypersensitivity and resistance to *C. neoformans*. Although nude mice displayed the ability to maintain cryptococcal population levels lower than did the phenotypically normal animals during the first 14 days of infection, the resistance was not sufficient to control the infection during the remainder of the 35-day experimental period. Heterozygote mice began to demonstrate positive delayed-type hypersensitivity responses by day 14 postinfection; however, nude mice were unable to mount delayed-type hypersensitivity responses. The appearance of the delayed-type hypersensitivity response in the heterozygote mice was concomitant with the reduced rate of proliferation of *C. neoformans* observed in those animals from days 14 to 35. Because anticryptococcal antibody titers and cryptococcal antigen levels were equivalent in both groups of mice, T-lymphocyte function was considered to be responsible for the resistance observed in the heterozygote mice. The mechanism by which cryptococcal populations were reduced was not addressed; however, the mouse model system used in these studies would be an ideal tool for studying those mechanisms. Nude mice were able to produce antibodies against cryptococcal cells, indicating that at least one component of *C. neoformans* is a T-independent antigen. The antibody response was predominantly immunoglobulin M in nude and heterozygote mice. Cryptococcal antigen levels were extremely high in both groups of animals and appeared to increase as *C. neoformans* cell numbers increased.

There is a considerable amount of circumstantial evidence which indicates that cell-mediated immunity plays an important role in defending the host against a *Cryptococcus neoformans* infection (1-3, 9, 11, 16, 17, 20, 21, 36, 38). Because T lymphocytes have been shown to be the mediators of resistance in cellular immunity in a number of other infectious disease systems (4, 6, 24, 33-35, 41), we decided to compare the pathogenesis of *C. neoformans* in a model deficient in mature T lymphocytes and a normal animal model. The athymic or nude mouse was chosen for the T cell-deficient system, and the heterozygote nu/+ mouse was chosen for the normal model. Because the nude mouse lacks T-cell activity, it would be expected, and has been demonstrated experimentally, that (i) delayed-type hypersensitivity (DTH) responses cannot be induced in nude mice (39) and (ii) antibodies can be elicited only if there is no requirement for T-lymphocyte participation with the B lymphocyte for induction (26).

The nude mouse model has been used in studying pathogenesis of a number of infectious agents which stimulate cell-mediated immunity and which are ultimately eliminated by the host by this mechanism. And, nude mice have been shown to possess heightened resistance when compared with normal mice after experimental infection with some of these agents such as *Listeria monocytogenes* (7, 14), *Brucella abortus* (7), *Salmonella typhimurium* (15), *Candida albicans* (37), or herpes simplex virus type 2 (29). The enhanced resistance was demonstrable only during the early stages of infection, whereas at the latter stages, in most instances, normal animals expressed more resistance. The innate early resistance of the nude mouse appears to be due to enhanced activity of macrophages found in athymic mice before experimental infection (7, 14, 15, 29, 32, 37, 42).

With these ideas in mind, the following questions were asked in this study. (i) Do nude mice

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show early enhanced resistance to a *C. neoformans* infection? (ii) How do T cell-deficient mice compare with normal mice in restricting proliferation of *C. neoformans* and in development of the DTH response to this organism? (iii) Are nude mice capable of producing immunoglobulins in response to *C. neoformans*, and if so are the immunoblobulins 2-mercaptoethanol sensitive? (iv) What are the cryptococcal antigen levels in infected animals throughout the course of disease?

MATERIALS AND METHODS

Organism. *C. neoformans* isolate 184, previously described (30, 31), was used throughout this study.

Animals. Female, 10- to 12-week-old congenitally athymic nude (nu/nu) mice of BALB/c background and phenotypically normal BALB/c mice heterozygous for the nude mutation (nu/+) were used in these experiments. The animals were obtained from Sprague-Dawley (Madison, Wis.) and were housed in sterile plastic cages with filter tops. Sterile water and mouse chow (Purina Lab Chow formula 5010 for autoclaving) were given ad libitum.

Antigen preparation. The antigen used for footpad testing was prepared by a modification of the method of Murphy and Cozad (30). Briefly, the procedure consisted of inoculating 1 liter of neopeptone dialysate broth with 10^9 viable *C. neoformans* 184 cells and incubating for 3 days at 30°C in a Fisher Psychrotherm incubator. *C. neoformans* cells were killed by adding Formalin to a final concentration of 2%. Then cells were removed by centrifugation, and the supernatant fluid was dialyzed against six changes of pH 7.2, sterile 0.15 M NaCl solution (SPSS). The dialyzed supernatant was sterilized by membrane filtration with a 0.45-μm filter (Millipore Corp., Bedford, Mass.). One liter of the supernatant was concentrated to 100 ml with an XM-50 membrane in an Amicon TCF10 thin-channel ultrafiltration system (Amicon, Lexington, Mass.). The concentrated material remaining in the chamber was washed by adding 100 ml of SPSS to the TCF10 chamber and again reducing the volume to 100 ml by filtration. The washing procedure was repeated three additional times. The washed, concentrated antigen was sterilized by membrane filtration and stored at −20°C until used. The protein concentration of this antigen (C-184) was 7.5 mg/ml as determined by the method of Lowry et al. (25) as modified by Miller (27). Total hexose concentration of C-184 antigen was 3.1 mg/ml as determined by the method of Dubois et al. (12).

Footpad testing. Footpad testing was accomplished by injecting 0.03 ml of C-184 antigen into the right rear footpad and an equal volume of SPSS into the left rear pad as a control (31). Engineer calipers (Mitutoyo Dial Calipers, Tokyo, Japan) were used to measure the thickness of footpads. Right (R_0) and left (L_0) footpads were measured before the injection of antigen or SPSS. At 24 h after injection of antigen or SPSS, right (R_{24}) and left (L_{24}) pads were measured again. The increase in footpad thickness for each mouse was determined by the following formula: $(R_{24} - R_0) - (L_{24} - L_0) = Z_{24}$. The arithmetic mean of the Z_{24} values was calculated for each group of mice. A mean Z_{24} value of 0.3 mm or greater was considered to be a positive footpad test.

Experimental procedure. Mice were injected intraperitoneally with 6.4×10^3 viable *C. neoformans* cells contained in 0.5 ml of SPSS. Viability of inoculum was determined by replicate spread plates prepared upon completion of inoculating the mice. At weekly intervals, for a total of 5 weeks, 4 to 6 nude and 4 to 6 heterozygote mice were footpad tested, bled by exsanguination for serum collection, and autopsied. The spleen, liver, lungs, and brain were collected aseptically and homogenized individually in sterile glass tissue grinder tubes with Teflon pestle tissue homogenizers type C (Arthur H. Thomas Co., Philadelphia, Pa.) containing 9 ml of SPSS. Appropriate dilutions of organ homogenates were plated in triplicate on Sabouraud dextrose agar and incubated at room temperature for 72 h. Colony counts were made, and the mean numbers of *C. neoformans* colony-forming units (CFU) per organ were calculated for each animal. Then the geometric means of CFU from each organ for each group of mice were calculated. Total CFU per animal was determined by summation of mean numbers of CFU in liver, spleen, lungs, and brain of each infected mouse. Then the geometric mean was determined for each group of mice.

Control animals consisted of nude and heterozygote mice inoculated intraperitoneally with 0.5 ml of SPSS, and they were treated in the same manner as *C. neoformans*-infected mice.

Serological tests. The whole-yeast cell microagglutination test for anticryptococcal antibody was employed (30). Rabbit anticryptococcal antiserum with a titer of 1:640 was used as a positive control, and normal rabbit serum was used as a negative control. In addition to determining anticryptococcal antibody titers on the nude and heterozygote mouse serum samples, each serum sample was treated with 2-mercaptoethanol by the procedure of Benedict et al. (5) as modified by Cozad et al. (8) before retesting in the microagglutination assay.

Cryptococcal antigen levels in sera were assayed with a cryptococcal latex agglutination kit purchased from International Biological Laboratories, Inc. (Rockville, Md.). Positive control for the latex test consisted of 0.25 μg of capsular polysaccharide per ml isolated from cultures of *C. neoformans*. Normal rabbit serum served as a negative control.

Statistical analyses. Means, standard error, and unpaired *t* tests programmed on a Hewlett-Packard calculator model 9810 A were used in analyses of data.

RESULTS

Pathogenesis of cryptococcosis in athymic and phenotypically normal BALB/c mice. At 7 days after injecting nu/nu and nu/+ mice with 6.4×10^3 viable *C. neoformans* cells intraperitoneally, the nude mice had fewer numbers of cryptococci in the four tissues cultured than did the nu/+ animals (Fig. 1a). Even though the *C. neoformans* cells were mul-

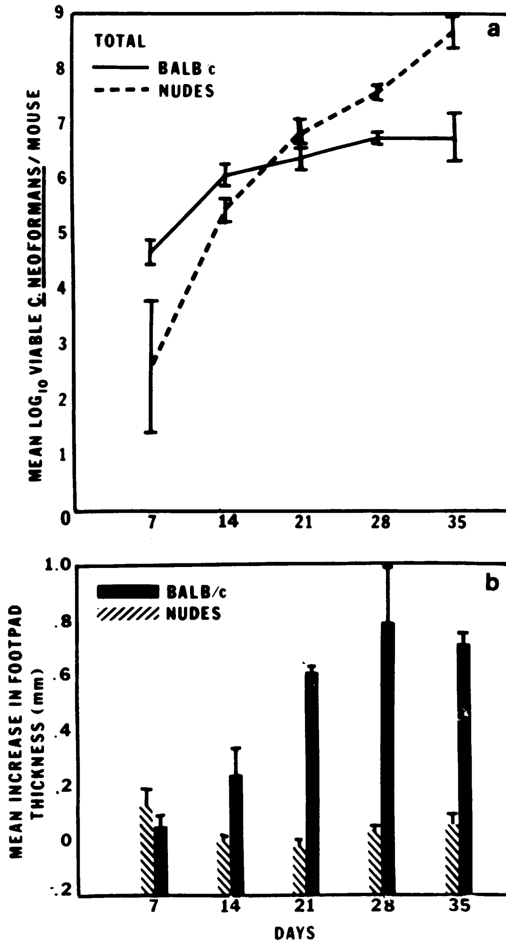

FIG. 1. *(a) Mean $\log_{10}$ viable C. neoformans as CFU cultured per mouse over the 35-day experimental period after infecting nude nu/nu or BALB/c, nu/+ mice with 6.4 × 10^3 viable C. neoformans cells. Each point represents the mean results for four mice. Vertical bars designate standard errors of the means. (b) Mean increase in footpad thickness 24 h after footpad testing the same animals described above with a cryptococcal culture filtrate antigen.*

tiplying faster in the nu/nu mice than in nu/+ mice during the next 7-day period, at 14 days postinfection athymic mice still had fewer organisms than did the infected normal BALB/c mice ($P < 0.05$). The phenotypically normal animals appeared to begin to gain control of the infection, because cryptococci numbers did not show a significant increase from day 14 postinfection throughout the 35-day experimental period. This was in contrast to the continuous rise in numbers of *C. neoformans* observed in the athymic animals. When numbers of cryptococci in nu/nu and nu/+ mice were compared on days 28 and 35, athymic mice had significantly higher numbers of organisms, with *P* values greater than 0.005 and 0.05, respectively.

Figure 2 shows the mean $\log_{10}$ viable *C. neoformans* counts in spleens, livers, lungs, and brains from the two groups of animals during the 35-day experimental period. The cryptococci population profiles in the individual organs reflected what was observed when the data were presented as a summation of *C. neoformans* CFU from the individual organs (Fig. 1). The reduced numbers of *C. neoformans* CFU observed in nude mice during the first 14 days of the experiment was most prominent in macrophage-ladened tissues such as spleens and livers (Fig. 2). In all organs from nude mice, the *C. neoformans* populations increased continuously throughout the experimental period, although in the normal mice there was evidence of *C. neoformans* populations decreasing in spleens, livers, and lungs during the last week of the experiment. The brains of nu/+ mice had fewer CFU than did brains of nude mice at day 35; however, the CFU counts in brains of normal mice did not appear to be decreasing, but rather they were increasing at a slower rate than in brains of athymic mice.

When individual organ CFU data obtained from the nude mice on days 28 and 35 were compared to equivalent data collected on nu/+ mice, it was found that nude mice had significantly higher numbers of CFU than the normal mice in all cases ($P < 0.05$).

DTH profiles. Infected nude mice did not develop DTH responses; however, the infected phenotypically normal mice did (Fig. 1b). Nude and normal uninfected control mice did not demonstrate increased footpad swelling in response to the cryptococcal antigen. In the infected nu/+ groups of animals, positive DTH responses began to appear 14 days after infection, and by day 21 100% of the infected nu/+ mice had positive footpad responses. This percentage of positive responders was also obtained at the two subsequent assay periods. The increase in mean DTH responses coincided with the reduced rate of increase in cryptococcal populations in the phenotypically normal mice (Fig. 1).

Serological data. Comparison of antibody data for the two groups of mice during the course of the disease is shown in Fig. 3. Some athymic mice produced anticryptococcal antibody, and usually the nude mice had higher titers than were observed in the phenotypically normal mice. Approximately 61% of the mice did not produce any detectable antibody. When mean antibody titers were calculated, nu/nu mice had slightly higher mean levels than the heterozy-

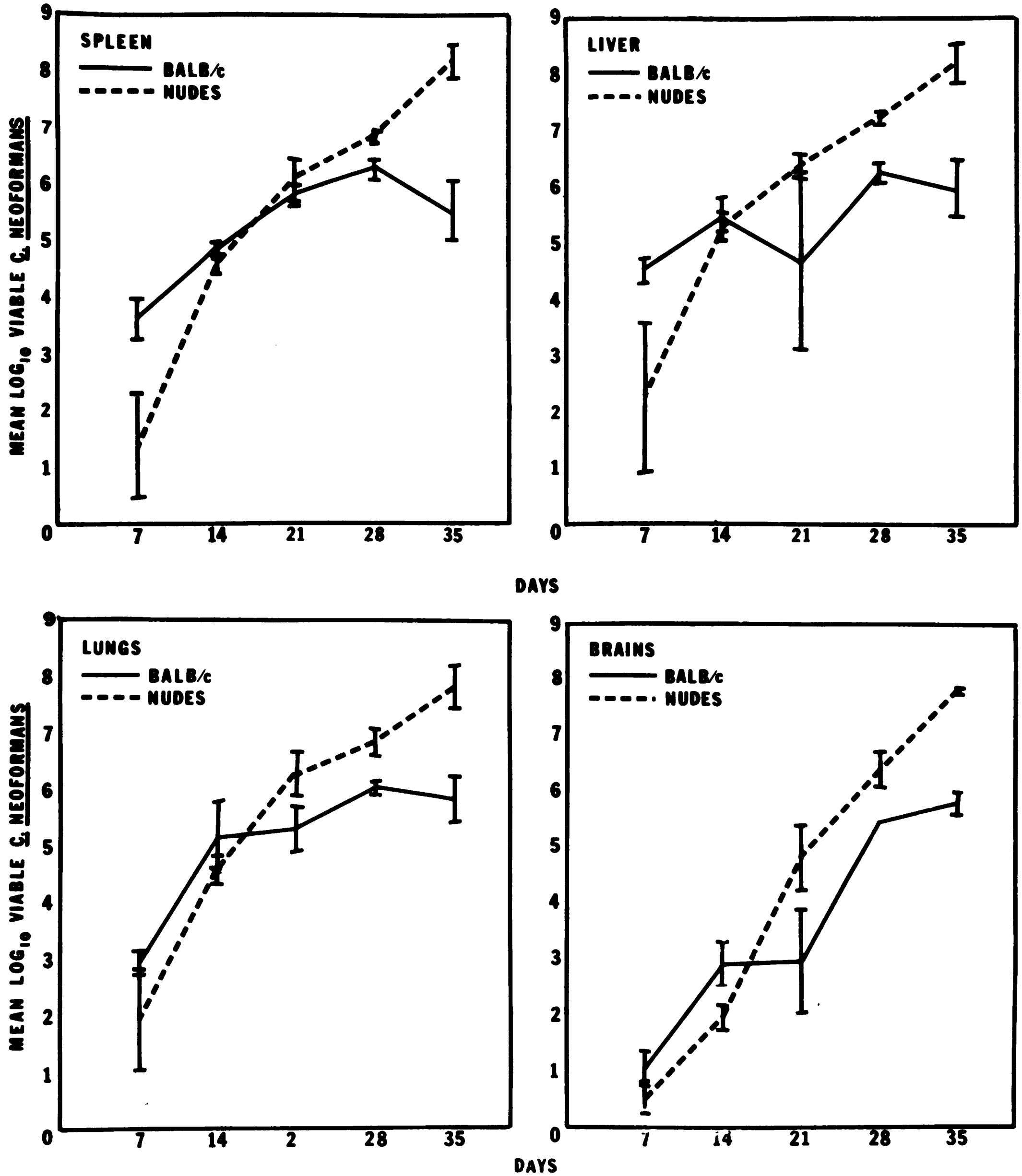

FIG. 2. *Mean log_{10} viable C. neoformans counts in spleens, livers, lungs, and brains of nude nu/nu and BALB/c, nu/+ mice infected with 6.4 × 10^3 viable C. neoformans cells. Each point represents the mean CFU counts of four mice. Vertical lines designate standard errors of the means.*

gotes at each time period except day 28; however, the unpaired Student's *t* test indicated that there were no significant differences in the two groups. Sera from control nude and heterozygote mice did not react in the antibody assay. To estimate the amount of immunoglobulin M antibody, nude and heterozygote serum samples were treated with 2-mercaptoethanol. Because this resulted in complete reduction of all positive agglutination titers to 0, the anticryptococcal antibody was considered to be essentially all immunoglobulin M.

Antigen titers in both groups of mice were extremely high, with mean log_2 values for nu/+ mice ranging from 13.5 to 14 and those for nude mice ranging from 15.3 to 19 (Fig. 4). The mean

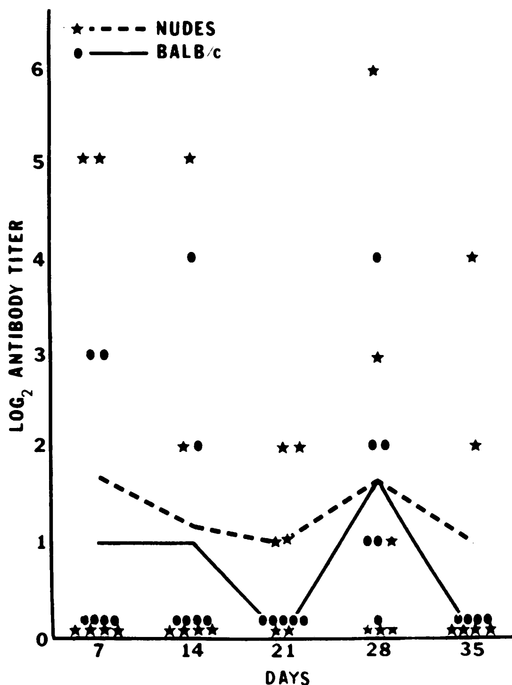

FIG. 3. *Anticryptococcal antibody titers of nude nu/nu and BALB/c, nu/+ mice injected with 6.4 × 10^3 viable C. neoformans cells. Each point represents the titer on one mouse. Interrupted line designates mean titers of nude, nu/nu mice. Solid line gives mean titers of BALB/c, nu/+ mice.*

antigen titers in the heterozygotes remained essentially the same throughout the course of the experiment, but the mean antigen titers in nude mice increased at days 28 and 35, corresponding to an increase in *C. neoformans* counts in those mice. Control sera from uninfected nude mice diluted 1:2 routinely weakly agglutinated the latex particles coated with anticryptococcal antibody; however, control serum from uninfected phenotypically normal BALB/c mice did not agglutinate the coated latex.

DISCUSSION

Athymic mice exhibited an early enchanced resistance to a *C. neoformans* infection. This was demonstrated by the fact that at 7 and 14 days after infection nude mice had fewer numbers of recoverable *C. neoformans* than did phenotypically normal BALB/c mice. Three factors lead us to propose that macrophages were responsible for this phenomenon. First, this observation was most evident in organs containing large numbers of phagocytic cells, namely, spleens, livers, and lungs. Second, other investigators have observed a similar innate mechanism when studying nude mice infected with a variety of pathogenic agents (7, 14, 15, 29, 32, 37); and in several instances (29, 32), it has been shown that nude mice possessed enhanced macrophage activity which contributed to the early clearing of the infectious agents. Third, macrophages can effectively phagocytize *C. neoformans* by way of a nonimmune defense mechanism as demonstrated by Mitchell and Friedman (28).

The early resistance seen in the nude mice was transient, and by day 28 postinfection athymic mice had substantially higher number of *C. neoformans* in spleens, livers, lungs, and brains than did the heterozygote mice. Because anticryptococcal antibody titers and cryptococcal antigen levels were equivalent in both groups of mice, the only difference in the two groups that can account for the heterozygote animals being able to gain control of the infection by day 28 is T-lymphocyte function. The heterozygotes

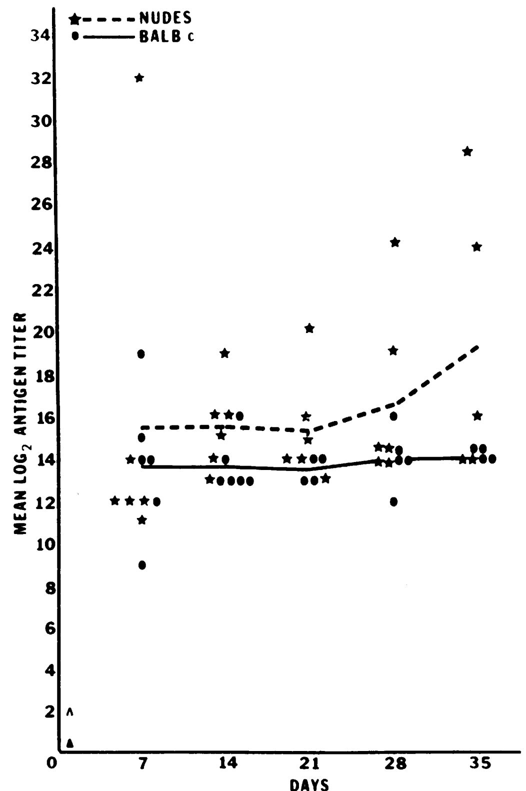

FIG. 4. *Cryptococcal antigen titers of nude, nu/nu and BALB/c, nu/+ mice infected with 6.4 × 10^3 viable cells of C. neoformans on day 0. Each point represents the antigen titer on one mouse. Interrupted line designates mean titers on nude mice. Solid line indicates mean titers of BALB/c, nu/+ mice. (△) and (▲) indicate the mean titers obtained on normal uninfected nude, nu/nu or BALB/c, nu/+ mice, respectively.*

were able to develop significant DTH responses, an assay of T-lymphocyte function, although the athymic mice were not. Furthermore, the rise in the DTH responses in the nu/+ mice was concomitant with the reduction of *C. neoformans* populations in spleen, livers, and lungs and with reduced rate of increase in *C. neoformans* populations in the brains. One infected athymic mouse died on day 30, and all four tissues examined contained large quantities of viable *C. neoformans*; however, during the course of the experiment, none of the infected heterozygote mice succumbed to the disease. Because there were not sufficient numbers of animals available to continue the experiment for a longer time or do survival studies, we cannot say whether the T-cell response was sufficient to completely eliminate the cryptococci from the heterozygote animals. But in another study done in our laboratory, by infecting CBA/J mice with approximately the same number of *C. neoformans* used in this experiment, we observed that the animals began to clear the cryptococci from the various tissues about the same time as in this study and the cryptococcal populations continued to be slowly reduced during the 92-day experimental period (unpublished data). In fact, some animals had no culturable cryptococci in the four tissues assayed and apparently had sterilized their infections (unpublished data). The results of the experiments with nude mice clearly show that without T-cell function mice did not develop a DTH response to cryptococcal antigen and were not able to control a cryptococcal infection as well as animals with normal T-cell function. Therefore, the T lymphocyte must be playing a role in host defense against *C. neoformans*. Both DTH responses and cell-mediated immunity require T lymphocytes as mediators (6, 13, 35, 41), but whether or not both of these functions of the T lymphocytes are activities of the same or different T-cell subpopulations is not known. Whether the same or different mechanisms are involved in the DTH response and the acquired resistance in cryptococcosis is another unknown. However, the mouse model as presented in this study has the potential to answer these questions.

Another conclusion which can be made from this study is that at least one antigenic determinant associated with *C. neoformans* can stimulate B cells to produce antibody without T-lymphocyte help. That athymic mice are able to produce anticryptococcal antibody supports this. The antibody produced by the athymic mice as well as heterozygote mice was essentially all immunoglobulin M, because 2-mercaptoethanol totally abolished the activity of the antisera. We could speculate that the antigenic determinant of *C. neoformans* responsible for stimulating the antibody production in the T-deficient model was probably the capsular polysaccharide of cryptococcus. T-independent antigens are usually antigens with repeating subunits of the same antigenic determinant (13) which cryptococcal polysaccharide is. Cryptococcal polysaccharide is somewhat similar to pneumococcal polysaccharide (23) which is a T-independent antigen (13). Production of only immunoglobulin M is another characteristic of thymus-independent antigens (13).

The immunoglubulin produced in nude mice against *C. neoformans* did not offer protection to those animals, and this reaffirms the work of Goren (19) indicating that antibodies are not protective in cryptococcosis. Antibody-dependent cell-medicated cytotoxicity, described by Diamond and Allison (10) as a possible mechanism for eliminating *C. neoformans* during the infection, was probably not significantly effective in our cryptococcosis model. Because antibody-dependent cell-mediated cytotoxicity activity to tumor cells has been shown to be higher in nude mice than in heterozygote animals (22), one might speculate that if antibody-dependent cell-mediated cytotoxicity was contributing to the elimination of *C. neoformans* then it would have been observed as lower numbers of *C. neoformans* CFU in the nude mouse group. However, we observed a continuous rise in *C. neoformans* CFU in the nude mice.

Cryptococcal antigen levels were extremely high in nude and heterozygote mice from day 7 after infection throughout the experimental period. In a similar study done in our laboratory with CBA/J mice (unpublished data), antigen titers were substantially lower. Because both positive and negative controls were run in the antigen assay since serum from uninfected mice did not react above a 1:2 dilution and because the same reagents were used on serum samples from the CBA/J mice and BALB/c mice, we have to assume that the reagents were good and the test was working properly. BALB/c and nude mice had at least 10 times more *C. neoformans* cells in their tissues than did CBA/J mice at comparable time periods; therefore, the level of polysaccharide could be reflecting the numbers of *C. neoformans* in the tissues. Another observation which supports this idea is that *C. neoformans* cell numbers in the nude mice rose concomitantly with antigen levels in those animals between days 21 and 35. For many years clinicians and medical mycologists have been aware that in cryptococcocis increasing antigen titers indicated increasing severity of disease and

vice versa (18, 40). Our observations support this.

The question arises as to why both antigen and antibody were present simultaneously in some animals in detectable amounts. There are two possible reasons for this. (i) The antigen being detected in the latex agglutination assay was predominantly cryptococcal polysaccharide. The antibody being detected by the whole-yeast cell agglutination test could have been reflecting several different populations of antibody molecules with different specificities. Therefore, the antibody that was being detected could have been antibody directed against antigenic determinants other than those being detected in the latex agglutination assay. (ii) Or possibly the antigen and antibody detected reflect the amount of free antigen and free antibody dissociated from antigen-antibody complexes (13) which were present in the mouse serum samples.

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