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Kinetics of Lymphocyte Transformation in Mice Immunized with Viable Avirulent Forms of *Cryptococcus neoformans*

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A murine model was developed to study the cell-mediated immune response of mice immunized with one of two live, avirulent forms of *Cryptococcus neoformans*: a nonencapsulated mutant and a thinly encapsulated pseudohyphal variant. A lymphocyte transformation assay was used to evaluate the cellular response of control and sensitized spleen cells after in vitro incubation with three merthiolate-killed whole-cell antigens of *C. neoformans*. An antigen-to-spleen cell ratio of 10:1 and 5 days of incubation of antigen-spleen cell mixtures were established as optimal conditions for maximum lymphocyte transformation. Maximum responses occurred from 2 to 3 weeks after the last of eight weekly intraperitoneal inoculations of *C. neoformans*. This assay provided an accurate, reproducible method of studying cell-mediated immunity to *C. neoformans*, and applications to the study of cryptococcal pathogenesis are proposed.

Cryptococcus neoformans is an encapsulated yeast which can cause subacute or chronic disease (3). Immunological responses of patients with cryptococcosis are minimal (9), and early attempts to demonstrate acquired resistance to cryptococcosis in animals immunized with killed suspensions of yeast cells (8, 14, 18, 20) and crude or purified capsular polysaccharide (8, 17) proved unsuccessful. More recently, some investigators have reexamined the concept of stimulating protective immunity in humans (1) as well as in animals (12, 15, 16).

Cell-mediated immunity is an important component of the immune response to mycotic infections, and increased susceptibility to cryptococcosis is associated with immunological defects (7). In a study of cell-mediated responses of patients who had recovered from cryptococcal disease, Graybill and Alford (11) observed diminished delayed-type hypersensitivity reactions to two commonly encountered fungi, *Histoplasma capsulatum* and *Candida albicans*. In a similar study, Diamond and Bennett (6) reported that in vitro lymphocyte transformation (LT) in response to cryptococcal antigen was lower in successfully treated patients than that of normal individuals who had positive skin tests.

The purpose of this investigation was to develop a murine model in which the cell-mediated immunity of animals immunized with live, avirulent strains of *C. neoformans* could be assayed by LT. The optimal LT kinetics reported here are being used in further studies of cell-mediated immunity in experimental cryptococcosis and will be reported later.

MATERIALS AND METHODS

Organism. *C. neoformans* NU-2-P is a thinly encapsulated, avirulent, pseudohyphal strain obtained by coculturing the virulent strain NU-2 (University of Nebraska Medical Center, Department of Medical Microbiology stock culture) with the soil amoeba *Acanthamoeba polyphaga* (21). Strain CIA is a moderately encapsulated yeast isolated from a patient with cryptococcal meningitis (22), and strain M7 is an avirulent nonencapsulated yeast which is an ultraviolet radiation-induced mutant of CIA (4). All strains are stock cultures at the University of Oklahoma Health Sciences Center.

Preparation of antigens. Live inocula of NU-2-P and M7 were grown on Sabouraud dextrose agar (Difco) at 25°C for 3 days. Cells were harvested by repeated washings with sterile saline (0.85%) and centrifugation at 10,000 × *g* for 10 min. Packed cells were resuspended in saline, and the concentration was adjusted to 10^5 cells per ml as determined by hemacytometer counts. Whole-cell antigens NU-2-P, M7, and CIA were grown as above: cells were killed by addition of 1:1,000 merthiolate for 24 h. Killed cells were washed 3× with saline and resuspended in RPMI-1640 (Grand Island Biological Co.). These antigens were adjusted to a concentration of 5×10^7 cells per ml (10:1 cryptococcal cell-to-lymphocyte ratio in the LT assay). Phytohemagglutinin (Difco) was rehydrated in RPMI-1640 and diluted 1:1,000 for the LT assay.

Animals. Eight-week-old, male C57/B16J mice (Jackson Labs, Bar Harbor, Maine) were used in the study. Two experimental groups received weekly intraperitoneal inoculations of 10^5 live NU-2-P and M7 cells per animal, respectively. A control group received

equivalent volumes of sterile saline.

LT assay. Lymphocyte transformation assays were initiated 1 week after the final intraperitoneal inoculation and were performed on randomly selected animals from each group for 4 consecutive weeks. The spleen cell preparation and LT assay methods employed were modifications of those reported by Blackstock et al. (2). Animals were sacrificed by cervical dislocation, the spleens removed aseptically and passed through a sterile screen (60 mesh). Splenocytes were washed 3× in Hanks balanced salt solution (Grand Island Biological Co.) and resuspended in RPMI-1640, supplemented with 5% heat-inactivated normal human serum, penicillin (100 U/ml), streptomycin (100 μg/ml), and Fungizone (0.25 μg/ml), to a concentration of 5×10^6 cells per ml as determined by hemacytometer counts. A 0.1-ml amount of the splenocytes was introduced into round-bottom microtiter plates (Linbro) for a final cell concentration of 5×10^5 cells.

To determine optimal in vitro incubation time, cell suspensions harvested at week 1 were divided into three cell groups for harvest at 3, 5, and 7 days, and quadruplicate cultures with each antigen were incubated at 37°C in a 5% CO_2 humidified atmosphere. At 18 h before harvest, tritiated thymidine (0.5 μCi; specific activity, 2 Ci/mmol) was added to each well. The cultures were harvested on glass fiber filters and prepared for counting by standard procedures. Samples were counted in a Packard C2425 liquid scintillation counter. Based on the results of this experiment, all subsequent LT assays were harvested after 5 days of incubation in vitro.

Statistical analysis. The transformation responses are expressed as the means of the quadruplicate samples plus or minus the standard error of the mean. The level of significance of the LT responses was determined by Student's *t* test for unpaired data.

RESULTS

Initially, a dose-response curve was established to determine optimal antigen concentration for LT. Spleen cells from mice inoculated intraperitoneally for 6 consecutive weeks with 10^5 viable NU-2-P cells gave maximum LT response in a 10:1 yeast cell-to-splenocyte ratio (Fig.1). This combination was used in all subsequent experiments.

Splenocytes from treated animals were tested against three strains of *C. neoformans* which differed in capsule thickness and in cellular morphology. Spleen cells from both groups of immunized mice showed statistically significant ($P < 0.01$) increases in their proliferative responses when incubated with each of three killed whole-cell antigens in comparison to saline controls (Table 1). There was no significant difference neither between the LT response of the two experimental groups to any antigen nor between the response of either group to the three cryptococcal antigens.

The LT responses of sensitized splenocytes incubated in vitro with antigen for 3, 5, or 7 days are illustrated in Fig. 2. The control bars express lymphocyte background (without antigen, unstimulated) counts per minute, and the phytohemagglutinin acts as a positive control showing that the spleen cells were viable and capable of

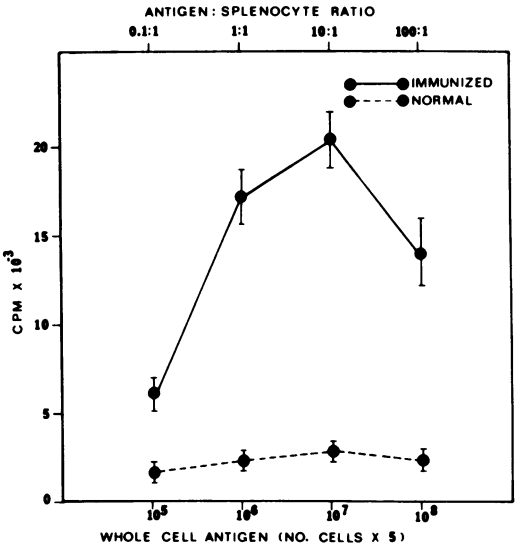

FIG. 1. *Dose response of killed, whole-cell NU-2-P antigen in LT assay. Data are from spleen cells pooled from six mice 1 week after six weekly inoculations of C. neoformans, strain NU-2-P. Vertical bars show standard error of the mean.*

TABLE 1. *Comparison of lymphocyte transformation counts per minute of spleen cells from animals 3 weeks after eight weekly inoculations of saline, M7, or NU-2-P cells of C. neoformans.*[a]

Immunization	Lymphocyte transformation (cpm ± SEM)[b]			
	Background[c]	NU-2-P[d]	M7[d]	CIA[d]
Saline	3,981 ± 552	4,110 ± 667	3,894 ± 541	4,211 ± 805
M7	4,220 ± 857	25,223 ± 2,994	27,871 ± 4,281	28,520 ± 3,316
NU-2-P	4,009 ± 746	26,884 ± 2,912	24,395 ± 2,291	22,673 ± 2,703

[a] Splenocytes were incubated in vitro for 5 days with one of three killed whole-cell antigens.
[b] SEM, Standard error of the mean.
[c] Means of three animals.
[d] Means of five animals.

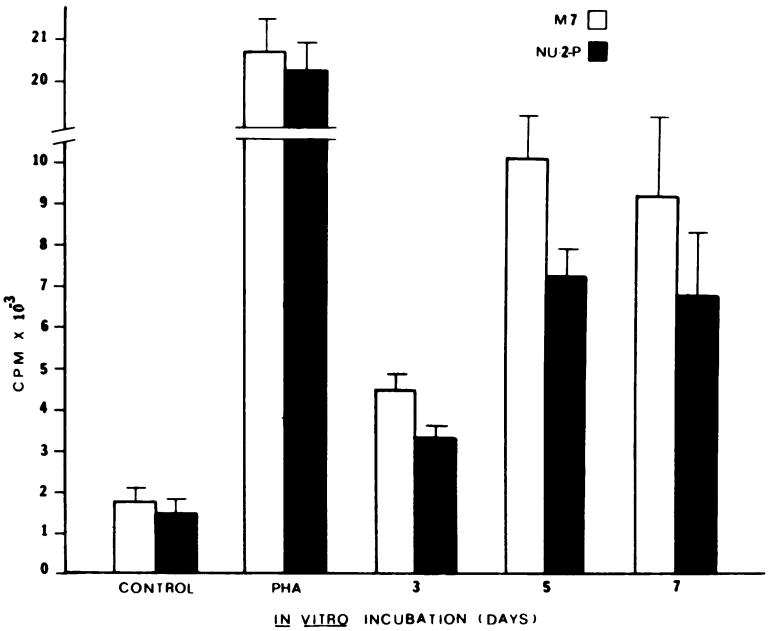

FIG. 2. *Lymphocyte transformation response of spleen cells taken from mice 1 week after eight weekly inoculations of M7 or NU-2-P strains of C. neoformans and incubated in vitro for 3, 5, or 7 days with killed, whole-cell NU-2-P antigen. Each bar represents mean counts per minute from five animals ± standard error of the mean. Control and phytohemagglutinin (PHA) results are 5-day incubations.*

proliferation. Maximum LT response was obtained with 5-day incubations. Seven-day incubations yielded a doubling of standard error; thus, 5-day incubations were chosen as optimal. Although spleen cells from mice immunized with strain M7 gave consistently higher counts per minute than NU-2-P-sensitized cells, the differences were not statistically significant ($P > 0.01$).

Figure 3 depicts the LT responses of splenocytes during the 4 weeks after eight inoculations. Maximum proliferation occurred at week 2 in mice immunized with strain M7 and at week 3 in mice immunized with strain NU-2-P. Responses between the M7- and NU-2-P-inoculated animals were not statistically significant ($P > 0.01$). Lymphocyte transformation responses showed a marked decrease in both experimental groups 4 weeks after final inoculation.

DISCUSSION

Immunization with cells or extracts of *C. neoformans* has been shown to produce positive delayed skin tests and prolonged survival (12). However, delayed-type hypersensitivity does not necessarily imply that protective immunity has been established since delayed-type hypersensitivity has been seen during active infection in animals that eventually succumb to the disease (12).

Reports in the literature are mixed as to the effectiveness of using capsular material or cells of *C. neoformans* for protection against infection with this fungus. In 1967, Goren and Middlebrook (10) described a procedure to elicit an antibody response to purified cryptococcal capsular polysaccharide which used polysaccharide-bovine gamma globulin conjugate as the immunogen. Although the antibody response to this challenge was significant, animals challenged with viable yeast cells were not protected. These results disagree with those of Louria et al. (19) who reported that enhanced resistance was correlated with the presence of antibody and not to an enhanced cellular response. Diamond and Allison (5) reported that several types of human peripheral leukocytes could participate in an antibody-dependent cellular killing. Interestingly, the one cell type which did not participate in the reaction was the T-lymphocyte. No in vivo correlates of this in vitro observation were reported.

Numerous investigators have suggested that increased susceptibility to crytococcal disease is associated with immunological defects, particularly cell-mediated deficiencies (3, 6, 7, 9, 11, 12). This has led to speculation that even when apparent underlying disease is not readily evident, subtile immune deficiencies may exist. Reports by Graybill and Alford (11) and Diamond

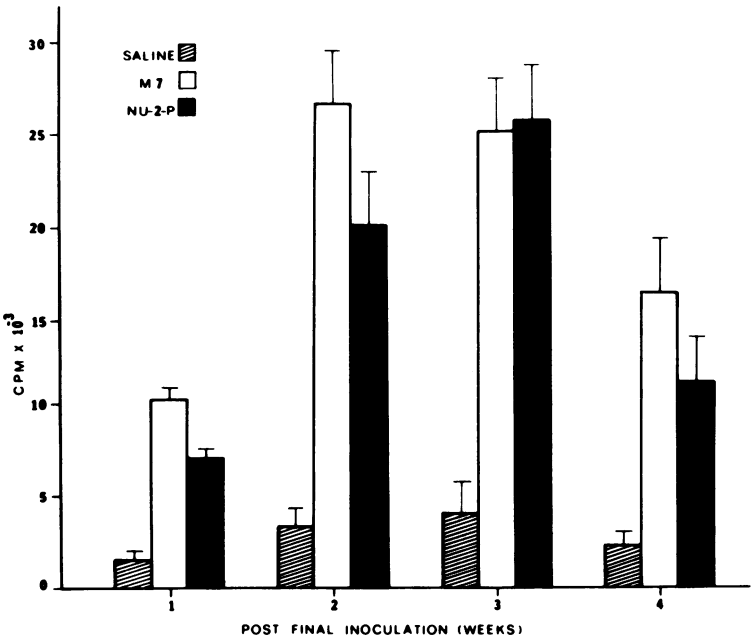

FIG. 3. *Lymphocyte transformation response of spleen cells taken from mice 1, 2, 3, or 4 weeks after eight weekly inoculations of saline (control), M7, or NU-2-P strains of C. neoformans. Cells were incubated in vitro for 5 days with killed, whole-cell NU-2-P antigen. Each bar represents mean counts per minute from three (control) or five (M7 and NU-2-P) animals ± standard error of the mean.*

and Bennett (6) have given support to the contention that cell-mediated immunity plays a major role in protection against *C. neoformans.*

We have developed an in vitro lymphocyte transformation assay for evaluation of the immune response to *C. neoformans.* The assay has been widely used in detecting lymphocyte responsiveness to other fungal antigens (13, 23) and has had limited use (6) in evaluation of immune responses to *C. neoformans* in humans. We have utilized intact, killed cryptococcal cells as an in vitro stimulant and have shown that a ratio of 10 killed cryptococcal cells per one splenocyte gives maximum stimulation in the LT assay (Fig. 1). This ratio provides adequate antigen concentration for maximal lymphocyte proliferation without causing decreased counts per minute due to antigen excess or toxicity. Optimal in vitro incubation of this mixture occurred at 5 days (Fig. 2). This observation agrees with those of Hall et al. (13) when immune responses to *Blastomyces dermatitidis* antigen were studied. Three days of incubation did not provide enough time for maximal responses, whereas 7 days of incubation produced substantial stimulation, but the standard error increased 100% over the 5-day data. This increased standard error may be due to the build-up of toxic metabolic products from the transforming lymphocytes which could interfere with optimal cell function.

No significant difference in stimulation was seen between the M7- and NU-2-P-sensitized spleen cells when exposed to killed antigen, indicating that both the nonencapsulated yeast (M7) and the thinly encapsulated pseudohyphal (NU-2-P) live-antigen inoculations stimulated host lymphocytes as detected by the in vitro LT assay (Table 1). Of particular note was the observation that there was no statistically significant ($P > 0.01$) difference in stimulation of sensitized cells against three different strains of *C. neoformans*: NU-2-P, M7, and CIA. The quantitative similarity of response to the three strains of killed antigen (Table 1) suggests that the lymphocyte stimulation is not due to capsular material, because M7 is not encapsulated and the other two strains are encapsulated. In addition, the presence of capsule does not appear to inhibit the LT response to the antigen that stimulates the lymphocytes (Mycopathologia, in press).

The magnitude of LT increased to a maximum at 2 to 3 weeks after 8 weeks of immunization, and then decreased at 4 weeks after the final inoculation (Fig. 3). There was no statistically significant difference ($P > 0.01$) between M7 and NU-2-P immunized animals, although in actual

counts per minute the M7 group peaked at week 2, whereas the NU-2-P group peaked at week 3. Both live inocula stimulated lymphocytes, which may indicate the development of immunity in cryptococcosis. Recent data suggest that eight weekly intraperitoneal inoculations with live, avirulent forms of *C. neoformans* without adjuvant will protect animals from subsequent intravenous challenge with virulent cells of *C. neoformans*. Additionally, the protection occurred when LT assays were positive (manuscript in preparation).

We have established a murine model in which one can consistently assay with accuracy the immune response of animals sensitized to *C. neoformans*. The kinetics of the LT assay have been established, and this procedure in being applied to further studies on the pathogenesis of cryptococcosis including immunization of experimental animals, evaluation of various lymphoid cell subpopulation functions, and protection of immunized animals from fatal challenge with virulent cells of *C. neoformans*.

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