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Transfer of Immunity to Cryptococcosis by T-Enriched Splenic Lymphocytes from *Cryptococcus neoformans*-Sensitized Mice

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Splenic enriched T-cells and sera were obtained from inbred CBA/J mice injected 7 or 35 days earlier with either 10^3 viable *Cryptococcus neoformans* or sterile physiological saline. The transfer of enriched T-cells collected 7 days after immunization or of normal enriched T-cells did not transfer immunity to *C. neoformans* or delayed-type hypersensitivity responsiveness to cryptococcal culture filtrate (CneF) antigen to the recipients. However, enriched T-cells harvested 35 days after immunization, when transferred to recipient mice, were able to confer immunity as indicated by the reduction in numbers of *C. neoformans* cells in the tissues, and they also transferred delayed-type hypersensitivity responsiveness to CneF antigen. Sera from either sensitized or normal mice were unable to transfer immunity to recipient animals. These results suggested that there was a time requirement for development of the immune response in the donor mice and that T-cells were crucial in the host defense against a cryptococcal infection. Culturing of day-35 *C. neoformans*-sensitized T-cells in the presence of homologous antigen (CneF) but not in the presence of heterologous antigen (purified protein derivative or 2, 4-dinitro-1-fluorobenzene) induced the production of migration inhibition factor, thus indicating that lymphocytes from *C. neoformans*-injected mice were specifically sensitized to CneF antigen.

Using a murine model, data have been generated which have clearly established that T-lymphocytes are necessary for effective host defense in cryptococcosis (5, 13, 14, 25). In providing protection, T-cells may function through cell-mediated immune mechanisms or by serving as helper cells in a humoral response. Immunological studies on host defense against cryptococcal infections have provided indirect evidence supporting cell-mediated immunity as the primary effective means of acquired host defense in this disease (1, 3, 5, 8-10, 12-15, 21, 25, 28, 32). Two studies have taken more direct approaches in attempts to show that cell-mediated immunity is the major mechanism of resistance. Abrahams et al. (2) reported that peritoneal leukocytes from *Cryptococcus neoformans*-immunized mice, when mixed with *C. neoformans* in subcutaneous air sac chambers, inhibited the multiplication of the cryptococci. Diamond (7) demonstrated that guinea pigs given *C. neoformans*-immune cells plus a challenge of viable cryptococci survived longer than infected control animals; however, the difference in survival times between the two groups was extremely small. The primary objective of this study was to establish the function of T-lymphocytes in protective immunity against a cryptococcal infection, by demonstrating that the transfer of T-lymphocytes, but not serum, from *C. neoformans*-sensitized mice could control the numbers of cryptococci in tissues of infected mice. This ability of sensitized T-lymphocytes to confer protection to recipient mice provides the evidence needed to establish that cell-mediated immunity plays an important role in host defense in cryptococcosis.

MATERIALS AND METHODS

Mice. CBA/J mice were bred in the animal facilities of the University of Oklahoma, using breeding stock purchased from Jackson Laboratory, Bar Harbor, Maine. Inbred mice of both sexes, 8 to 12 weeks of age, were used in the study.

Organism. *C. neoformans* 184A as described by Murphy and Cozad (22) was employed throughout this study.

Immunization of mice. After ether anesthesia, mice were immunized intranasally with approximately 10^3 viable yeast cells in two 5-μl saline droplets dispensed from a 50-μl Hamilton glass syringe attached to a 27-gauge needle. The exact number of viable *C. neoformans* given per mouse varied slightly from one experiment to another; these numbers ranged from 1.52×10^3 to 1.69×10^3 cells with a mean of 1.6×10^3. Control mice were similarly treated but given 10 μl of sterile physiological saline solution.

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Complement. Guinea pig serum for use as complement was absorbed with CBA/J mouse erythrocytes, liver cells, and spleen cells according to the procedure of Reif (30). Briefly, the procedure was as follows. Guinea pig serum was added to mouse erythrocytes in a ratio of 20 volumes of serum to 1 volume of packed cells. The cell suspension was mixed gently and allowed to rest on ice in a 4°C refrigerator for 30 min. After centrifugation at 5°C for 10 min at 1,000 × *g*, the serum was collected and added to fresh erythrocytes, and the absorption process was repeated. The supernatant was then absorbed three times with 1 volume of packed mouse liver cells to 10 volumes of supernatant. Fresh mouse liver cells were used for each absorption. Finally, the supernatant was absorbed with 1 volume of packed mouse spleen cells for each 20 volumes of supernatant. The absorbed guinea pig complement (AGPC) was dispensed into tubes in 3-ml volumes and stored at −20°C until needed. When the AGPC was assayed, undiluted, with normal mouse serum on CBA/J thymocytes, spleen cells, or splenic T-enriched cell pools, the percentage of killing ranged from 0 to 6%.

Antiserum. Anti-thy 1.2 antiserum was prepared by immunizing AKR/J mice with approximately 10^7 viable CBA/J mouse thymocytes according to procedures described by Reif and Allen (31). A block titration procedure was employed to evaluate the cytolytic activity of the anti-thy 1.2 antiserum. The assay was comprised of three components, the anti-thy 1.2 antiserum, AGPC, and CBA/J thymocytes (5.0×10^7 cells per ml); each component was added in 0.1-ml volumes. Eight sets of twofold serial dilutions of anti-thy 1.2 antiserum were prepared using Dulbecco phosphate-buffered saline as the diluent. Duplicate AGPC controls were prepared by adding 0.1 ml of normal mouse serum in place of anti-thy 1.2 antiserum. The appropriate volume of AGPC was added to each tube such that duplicate sets of antiserum dilutions and AGPC control tubes had a final complement concentration of 10, 20, 33, or 40%. CBA/J thymocytes were added to each of the assays before incubation for 2 h at 37°C. To evaluate the amount of cell killing, the trypan blue dye exclusion test was used. A 1:128 dilution of the anti-thy 1.2 serum with 33% AGPC killed 50% of the CBA/J thymocytes. With other concentrations of AGPC, the dilution of anti-thy 1.2 serum showing 50% killing was one to two tubes lower than with the 33% AGPC. In AGPC controls there was 0 to 5.7% killing of thymocytes. To achieve a 98% killing of thymocytes, a 1:4 dilution of the anti-thy 1.2 antiserum was required in the presence of 33% AGPC; therefore, in all experiments in which lymphocyte pools were treated with anti-thy 1.2 and complement to eliminate T-lymphocytes, these concentrations of reagents were used. Treatment of lymphocytes with antiserum and AGPC was done at 37°C for 2 h, followed by three washings with Dulbecco phosphate-buffered saline before suspending the cells in appropriate volumes of Dulbecco phosphate-buffered saline for injection into recipient mice. When normal CBA/J spleen cells were treated with anti-thy 1.2 plus AGPC under the conditions described above, the mean percent killing achieved was 28.3% (range, 26.5 to 29.1%). Since approximately 65% of the spleen cells are B-cells and 35% are T-cells (4, 11), the percent killing achieved with the anti-thy 1.2 serum was within the expected range. When T-enriched cell pools were treated with anti-thy 1.2 antiserum and AGPC, as described above, killing ranged from 94 to 99.2%, values expected for T-enriched populations. Considering all of these results, we concluded that the anti-thy 1.2 serum that had been prepared according to a standard procedure was killing T-lymphocytes specifically.

Collection and enrichment of T-lymphocytes. Splenic cells from immunized and saline-treated control mice were harvested according to the procedures described previously (19). Enriched T-cell populations were obtained using the nylon wool method of Julius et al. (18). Approximately 3.0×10^8 spleen cells were applied to a 0.7-g nylon wool column, and on an average 32% of the cells were recovered in the filtrate. To determine the amount of B-cell contamination in the T-enriched spleen cell pools, an immunofluorescent staining procedure using fluorescein-labeled goat anti-mouse immunoglobulin (Cappel Laboratories, Cockranville, Pa.) and a cytotoxicity assay using AKR/J anti-CBA/J thy 1.2 antiserum plus complement were performed. Each assay procedure was repeated five times on each nonadherent cell pool. The mean percentage of immunoglobulin-positive cells was 7.5% (range, 5 to 11%). The mean percentage of viable cells after treatment with anti-thy 1.2 and complement was 3.5% (range, 0.8 to 6%). The results of both assays indicate that approximately 95% of the cells in the T-enriched pools were T-lymphocytes. The viability of spleen cells after separation was greater than 97% as determined by the trypan dye exclusion test.

DTH response. On 7 and 35 days after intranasal administration of *C. neoformans*, mice were randomly selected and footpad tested for delayed-type hypersensitivity (DTH) responsiveness to cryptococcal culture filtrate antigen (CneF) according to the procedure described by Cauley and Murphy (5).

Transfer of lymphoid cells. On days 7 and 35 after the intranasal administration of 1.5×10^3 viable *C. neoformans* cells or saline, mice were put under deep ether anesthesia. Blood was obtained through cardiac puncture, and sera were collected, pooled, and stored at −20°C until needed. Spleens were removed from donor mice for transfer of spleen cells or for obtaining enriched T-lymphocyte populations. Recipient mice were divided into four groups, and all groups were challenged with approximately 10^4 viable *C. neoformans* cells intravenously. The numbers of viable *C. neoformans* injected varied slightly from experiment to experiment, ranging from 1.2×10^4 to 1.8×10^4 viable cells. Two hours after *C. neoformans* challenge, lymphocytes were administered intravenously. Group 1 mice were given 0.2 ml of saline. Group 2 mice received 10^8 sensitized spleen cells or sensitized T-lymphocytes in 0.2 ml of sterile physiological saline solution, depending on the experiment. Group 3 mice received 10^8 sensitized enriched T-lymphocytes which had been treated with anti-thy 1.2 antiserum plus complement to eliminate the T-cells. Group 4 mice were given 10^8 spleen cells or enriched T-lymphocytes obtained from normal saline-treated mice. Approximately 20 h after lymphocyte transfers, five mice from each group were selected randomly and footpad tested

to determine the DTH responsiveness to CneF antigen. The remaining mice were autopsied for counts of *C. neoformans* colony-forming units (CFU) at various time periods after cell transfers.

Serum transfer. "Immune" and normal sera obtained from *C. neoformans*-sensitized and nonsensitized mice, respectively, were used for transfer into recipient mice. Two groups of mice were intravenously injected with 1.3×10^4 viable *C. neoformans* cells, 2 h before the injection of serum. One group received intravenously 0.5 ml of serum from nonsensitized mice, whereas the other group received 0.5 ml of serum from sensitized mice. The same volume of the appropriate serum was administered again on days 3, 6, and 10 postchallenge to those mice which had not been sacrificed during the previous autopsy period. Twenty hours after the first serum transfer, five mice from each group were footpad tested with CneF antigen. The remaining mice in each group were autopsied at various time periods after serum transfers to determine the counts of *C. neoformans* CFU.

Viability counts for *C. neoformans*. One day after the injection of lymphocytes or serum, three mice from each group were sacrificed, and spleens, livers, lungs, and brains were collected. The numbers of viable *C. neoformans* cells in each organ were determined according to the procedure described by Cauley and Murphy (5). Similarly, autopsies were performed on days 3, 7, 10, and 14 posttransfer of lymphocytes and on days 7 and 14 after serum transfer.

Macrophage migration inhibition test. The macrophage migration inhibition test was used to demonstrate that the sensitized lymphocytes were specifically sensitized to *C. neoformans*. Mice were subjected to one of the following: (i) 10^3 viable *C. neoformans* given intranasally; (ii) 0.5% 2,4-dinitro-1-fluorobenzene (DNFB) (Sigma Chemical Co., St. Louis, Mo.) applied according to the procedure of Phanuphak et al. (29); (iii) 0.5 ml of complete Freund adjuvant (Difco), injected subcutaneously on the back and on the left and right sides of the abdomen, followed 1 week later by another 0.1 ml of complete Freund adjuvant given intraperitoneally; or (iv) sterile physiological saline solution, given to a group of control mice. Thirty-five days after *C. neoformans* immunization, 4 days after the last skin painting with DNFB or 7 days after the last injection of complete Freund adjuvant, four mice from each of the respective groups and from the saline control group were sacrificed. Spleens from these mice were removed and enriched for T-lymphocytes as described earlier. Enriched T-cells were adjusted to a concentration of 5×10^6 cells per ml in Medium 199 containing 27 mM sodium bicarbonate, 50 U of sodium penicillin G, and 50 μg of streptomycin per ml and supplemented with 5% fetal calf serum. Each T-lymphocyte-enriched pool was dispensed in 2-ml quantities into each of eight plastic tissue culture tubes (16 by 25 mm, Falcon Plastics). To two tubes of each enriched T-lymphocyte pool was added one of the following: (i) 100 μl of CneF antigen having a predetermined optimal concentration of 100 μg of protein; (ii) 100 μl of 0.05% DNFB in saline (vol/vol); (iii) 100 μl of a 25-μg/ml solution of purified protein derivative (Parke Davis & Co., Detroit, Mich.); or (iv) 100 μl of sterile physiological saline solution. The final volume in each tube was adjusted to 2.5 ml by adding 100 μl of a suspension of 3×10^4 mineral oil-induced peritoneal exudate cells per ml and 0.3 ml of Medium 199. The tubes were incubated for 48 h at 37°C in 5% CO_2–95% air. After centifugation at 750 × *g* for 10 min, supernatants were collected, and migration inhibition factor (MIF) activity was determined using the procedure described by Harrington and Stastny (17). Mineral oil-stimulated macrophages from normal CBA/J mice were used as target cells.

Statistical analyses. The mean, standard error, and unpaired *t* test, programmed on a Hewlett Packard model 9810A calculator, were used in analyses of the data.

RESULTS

DTH responses. Donor mice immunized intranasally with viable *C. neoformans* 7 days earlier showed negative DTH responses to CneF antigen; however, by day 35 postinfection, donor mice demonstrated strong DTH responses to CneF antigen (Fig. 1). Recipient mice which had received either sensitized, nonsensitized, or sensitized enriched T-cells pretreated with anti-thy 1.2 antibody plus complement from day-7 donor mice did not have positive responses to CneF antigen when footpad tested 20 h after cell transfer. In contrast, mice that had received sensitized enriched T-cells from day-35 donors showed positive DTH responses. Other groups of mice given day-35 nonsensitized or day-35 sensitized T-cells treated with anti-thy 1.2 plus complement did not develop DTH responses to CneF antigen (Fig. 1). When the results of this experiment were subjected to statistical analysis, only recipients of untreated sensitized T-cells had significantly greater footpad responses than any other group ($P < 0.005$).

Effects of cell transfers on resistance. Several experiments were performed to evaluate the potential of lymphoid cells from *C. neoformans*-sensitized mice to transfer resistance to a cryptococcal challenge injection. The first set of experiments followed the same protocol as for passive transfer of the DTH response; however, 1.5×10^8 spleen cells were transferred, and instead of determining footpad reactivity, the recipients were challenged intravenously with 1.6×10^4 viable *C. neoformans* cells. Three mice were randomly selected from each group on days 1, 3, 7, 10, and 14 postchallenge and were autopsied to determine the numbers of CFU per organ. The results from these experiments showed a reduction in numbers of cryptococcal CFU in organs from recipient mice which had received spleen cells collected 35 days after immunization, as compared to cryptococcal CFU in organs from recipients of control spleen cells or of spleen cells harvested 7 days post-immunization.

The next set of experiments was designed to

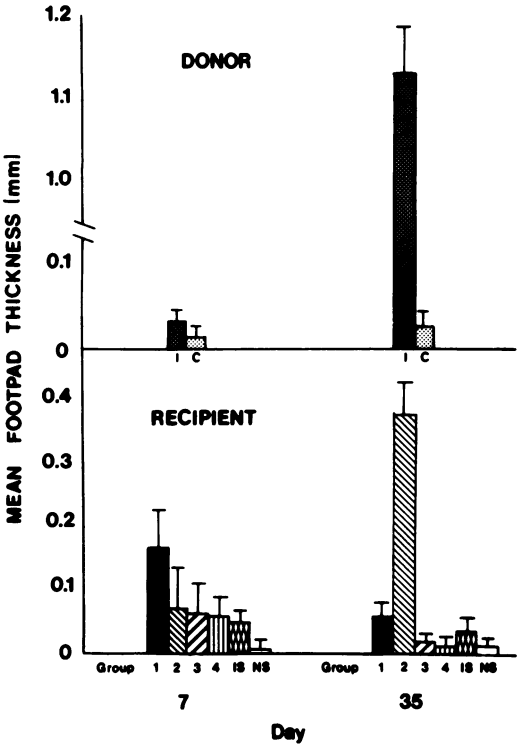

FIG. 1. *DTH responses to CneF antigen in footpads of donor mice and their lymphoid cell recipients. I, Immunized mice; C, saline-treated mice. Group 1 received 1.8 × 10⁴ viable C. neoformans. Group 2 received 1.8 × 10⁴ viable C. neoformans and 1.0 × 10⁸ enriched T-cells harvested from donors sensitized 7 or 35 days previously. Group 3 received 1.8 × 10⁴ viable C. neoformans plus 10⁸ sensitized, enriched T-cells treated with anti-thy 1.2 and AGPC, collected from day-7 or day-35 donors. Group 4 received 1.8 × 10⁴ viable C. neoformans and 10⁸ normal enriched T-cells. IS, Mice that received serum from immunized donor mice; NS, mice given serum from saline-treated donor mice. Footpad testing in recipients was done 20 h after transfers. Vertical bars designate the standard errors of the means.*

show that the T-enriched fraction of sensitized spleen cells was responsible for the passive transfer of protection. These experiments were done twice. The protocol described for transfer of spleen cells was employed; however, T-enriched populations were used in place of spleen cells. In experiment 1, the challenge dose of *C. neoformans* cells was 1.8×10^4 viable cells; in experiment 2, it was 1.2×10^4 viable cells. The results of both experiments were similar. Figure 2 shows the data from experiment 1. Seven days postimmunization donor cells were unable to passively transfer resistance, as indicated by the fact that there were no significant differences among the four transfer groups (Fig. 2A). However, a different picture was observed in recipients which had received enriched T-cells from donor mice infected with *C. neoformans* 35 days previously (Fig. 2B). Although the mean numbers of *C. neoformans* CFU among the four transfer groups were not significantly different 1 day after cell transfers and challenge, by day 3 mice that had been given *C. neoformans*-sensitized enriched T-lymphocytes had lower numbers of culturable *C. neoformans*, with mean CFU per mouse of 2×10^3 as compared to approximately 3×10^4 CFU per mouse obtained in each of the other three experimental groups (Fig. 2B). Increases in mean numbers of *C. neoformans* CFU were observed in all 35-day recipient groups throughout the remaining 14-day assay period, but group 2 mice maintained significantly lower numbers of CFU than any of the other groups ($P < 0.005$).

Effects of serum transfers. The mean numbers of *C. neoformans* CFU were essentially the same for the mice that received immune donor serum and for mice that received control donor serum, whether the serum came from donors 7 or 35 days after sensitization or saline treatment (Fig. 3). Even though one group of recipients, autopsied 14 days postchallenge, had been given three immune serum injections, they did not show any reduction in numbers of *C. neoformans* CFU as compared to control recipients.

Migration inhibition test. To determine the degree of specificity of the sensitized T-lymphocytes from *C. neoformans*-sensitized mice, a migration inhibition assay was employed using CneF antigen and two unrelated antigens, purified protein derivative and DNFB. Results of this study are presented in Table 1. Sensitized lymphocytes obtained from mice immunized with *C. neoformans*, complete Freund adjuvant, or DNFB did not produce significant amounts of MIF when cultured in the presence of the heterologous antigens: however, significant levels of MIF were produced in the presence of the homologous antigens. *C. neoformans*-sensitized T-cells stimulated with CneF gave results that were significantly different from results obtained by stimulating the same cells with purified protein derivative or DNFB with $P < 0.001$.

DISCUSSION

The data presented in this study provide strong direct evidence for the contention that cell-mediated immunity is the primary means of host defense in cryptococcosis. Using a murine model rather than guinea pigs, we have affirmed Diamond's (7) results indicating that resistance to *C. neoformans* could be conferred on recipient animals by passively transferring lymphoid cells, but not serum from sensitized donor animals.

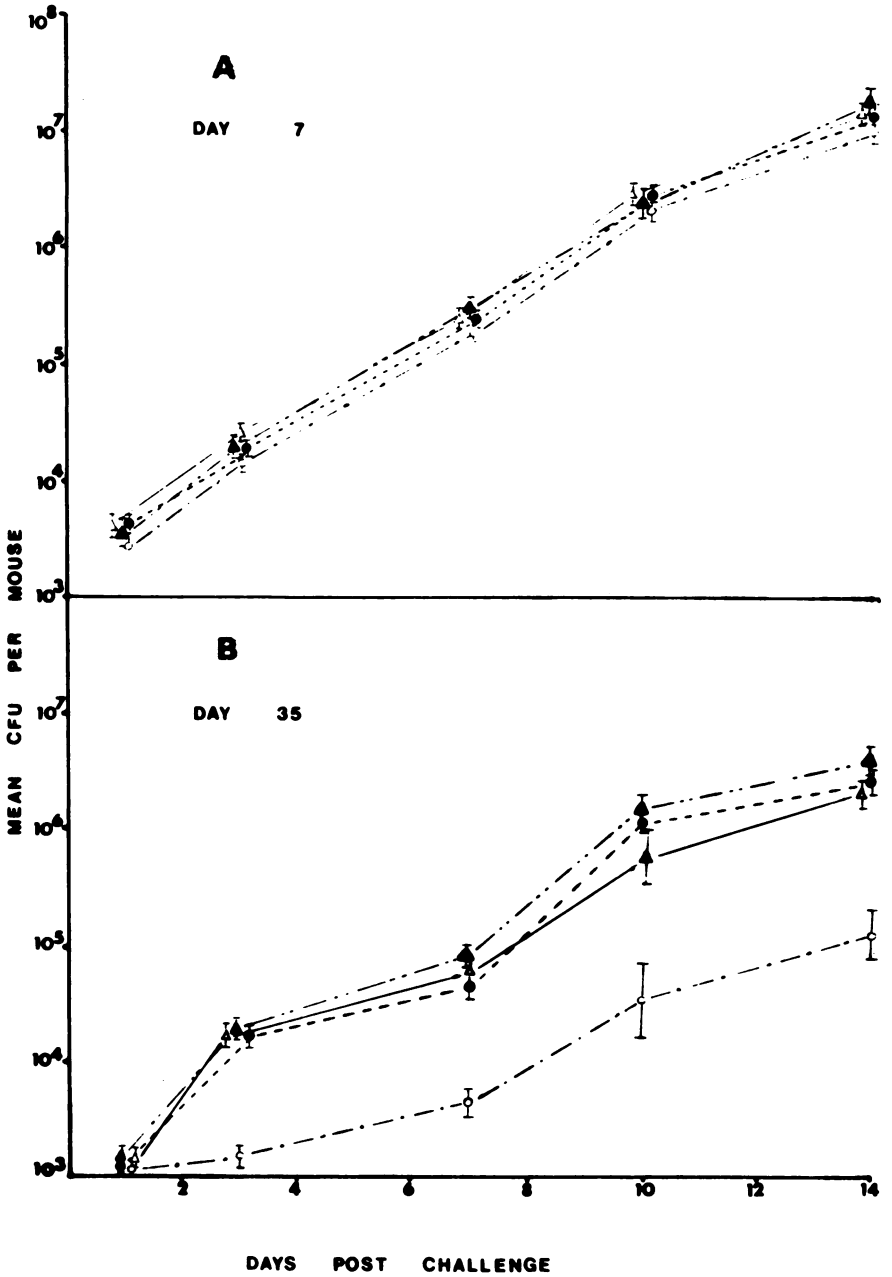

FIG. 2. *Mean numbers of C. neoformans CFU cultured at various times from recipient mice which had been injected intravenously with 10^4 viable C. neoformans cells on day 0. Lymphoid cells for transfer were obtained (A) 7 days or (B) 35 days after treatment of donors. Group 1 (△——△) received no lymphoid cells. Group 2 (○— - — ○) was given 10^8 enriched T-cells from C. neoformans-sensitized donors. Group 3 (●- - -●) received 10^8 T-enriched spleen cells treated with anti-thy 1.2 plus complement and group 4 (▲——▲) was injected with 10^8 normal T-enriched spleen cells. Vertical bars designate the standard errors of the means.*

Furthermore, we have demonstrated that T-lymphocytes in the immune population of lymphoid cells are the cells responsible for resistance. This was shown in two ways in this study. First, depleting or greatly reducing the numbers of B-cells and other adherent cells, such as macrophages, from the sensitized spleen cell pool did not affect the ability of the cells to passively transfer resistance, but, in fact, enhanced the transferred resistance. Second, the ability of the

sensitized enriched T-cell pools to transfer immunity could be abolished by killing the T-lymphocytes with anti-thy 1.2 antiserum and complement. The mechanism by which the T-cells reduced the numbers of cryptococci in tissues was not addressed in this study; however, some observations were made with respect to the amount of time required after sensitized T-cell transfer before the killing effects on *C. neoformans* could be noted. By day 3 after immune cell transfers, the effect sensitized T-cells had on *C. neoformans* cell numbers could be observed in recipient animals (Fig. 2B). This was comparable to reports of similar studies done with tuberculosis in which 7 days lapsed after immune cell transfer before effects could be observed in organism numbers (19).

Using various cryptococcosis models, we (5, 21; R. A. Bates and J. W. Murphy, Abstr. Annu. Meet. Am. Soc. Microbiol. 1975, F26, p. 89) as well as others (7, 15) have noted a parallel in the development of the DTH responsiveness to cryptococcal antigens and in the acquisition of resistance to a cryptococcal infection. In this study we have shown that the same T-lymphocyte cell pool that could transfer DTH responsiveness could also transfer resistance. In addition, that T-cell pool contained cells capable of responding specifically to cryptococcal antigen with the production of MIF. These three parameters could be activities of a single cell population; however, most likely, the immune T-cell pool used in these studies was a heterogeneous population containing several different subsets of T-lymphocytes. We did not determine whether or not the three parameters measured, namely, DTH, resistance, and MIF production, were properties of the same T-cell subset or represented activities of two or three subsets which develop simultaneously after appropriate antigenic stimulation. In other infectious disease systems such as listeriosis (16, 26) and tuberculosis (20, 27), it has been shown that effective immunity and DTH responsiveness are not always coexisting features. Further studies must be done to resolve this question in murine cryptococcosis.

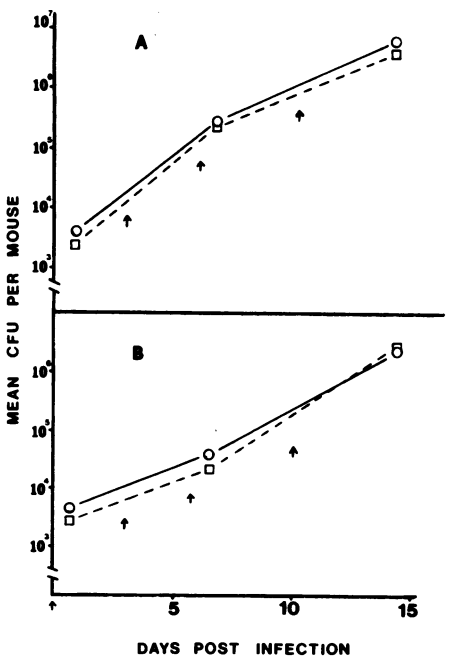

FIG. 3. *Mean numbers of C. neoformans CFU isolated from recipient mice which had been challenged intravenously with 1.3 × 10⁴ viable C. neoformans, then given serum collected from donor mice immunized 7 days (A) or 35 days (B) previously with 10³ viable C. neoformans* (□) *or saline* (○). ↑ *indicates serum injections.*

TABLE 1. *MIF production by sensitized lymphocytes after antigen stimulation*

No. of immune mice	Immunizing agent	% Migration inhibition[a]		
		CneF[b]	DNFB[b]	PPD[b]
4	*C. neoformans*	56	10	12
4	DNFB	12	45	18
4	Complete Freund adjuvant	4	12	46
4	Saline	2	5	1

[a] Percent migration inhibition = [1 − (test migration distance/control migration distance)] × 100. PPD, Purified protein derivative.

[b] Stimulating antigen.

There is an additional facet of this study which should be mentioned. The results of the MIF studies demonstrated that the CneF antigen was quite effective in stimulating *C. neoformans*-sensitized lymphocytes to produce MIF. Furthermore, the antigen did not significantly enhance or inhibit migration of macrophages in control systems, and its ability to stimulate MIF was specific. This was expected since the CneF antigen has been shown to have a high degree of specificity when used to elicit DTH responses (23, 24). In general, MIF production has been shown to be a good in vitro correlate of DTH responsiveness (6). Considering all of these features, the CneF antigen appears to be an excellent reagent for a MIF assay for specifically detecting sensitivity to *C. neoformans*.

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