In Vitro Interactions of Immune Lymphocytes and *Cryptococcus neoformans*

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CBA/J mice immunized subcutaneously with emulsions of heat-killed *Cryptococcus neoformans* in complete Freund adjuvant displayed delayed-type hypersensitivity to cryptococcal culture filtrate antigen and developed sensitized splenic lymphoid cells which inhibited the growth of *C. neoformans* in vitro. The in vitro assay of growth inhibition served to investigate further the kinetics of the effect of sensitized lymphoid cells on the pathogen. There was a close correlation between the delayed-type hypersensitivity response in mice and inhibition of growth of *C. neoformans* by lymphoid cells. Sensitized splenic lymphocytes capable of inhibiting the growth of the cryptococci were detected at day 6 after immunization and reached maximum levels by days 8 through 16. Inhibition of growth was highest with effector-to-target cell ratios of 300:1 or greater. Inhibition of growth of *C. neoformans* by sensitized lymphoid cells was detectable as early as 4 h after effector and target cells were mixed and increased gradually, reaching a maximum at 24 h, but dropped significantly by 48 h. By supplementing the reaction mixtures with fresh medium or additional sensitized effector cells during incubation, the inhibition of growth of *C. neoformans* could be maintained through 48 h. *C. neoformans*-sensitized effector lymphoid populations not only inhibited the growth of the pathogen in vitro but also restricted *C. neoformans* proliferation in various vital organs upon transfer to naive recipient animals, indicating that the in vitro growth inhibition assay may be a means of assessing the resistance of animals to *C. neoformans*. The effector cells from sensitized animals were nylon wool-nonadherent Thy-1⁺ and Ia⁺ lymphocytes.

Cryptococcosis is a mycotic disease caused by an encapsulated yeast-like fungus, *Cryptococcus neoformans*. The route of infection in humans and animals is believed to be through inhalation of the small yeast particles, but inhalation of basidiospores, which are the spores of the perfect stage of the fungus (*Filobasidiella neoformans*), has also been suggested recently as a possible means of infection (16, 30, 47).

Several host defense mechanisms effective against cryptococci have been described. Both humoral and cell-mediated immune mechanisms have been implicated (1, 2, 4, 8, 10-14, 19-21, 24, 26, 32, 33, 35, 38, 51). In recent years, more and more evidence has accumulated indicating that cell-mediated immunity is the primary means of host defense (1, 2, 8, 12, 19-21, 32, 33). The murine model has provided insights into the interactions of the immune system of the host with *C. neoformans* (32, 33). The studies of *C. neoformans* infections in the athymic mouse model substantiated the importance of the role

of thymus-dependent (T) lymphocytes in protection against the infection (8, 20). Lim and Murphy (32) recently showed that the delayed-type hypersensitivity (DTH) response to cryptococcal culture filtrate (CneF) antigen as well as resistance to further challenge with viable cryptococci could be adoptively transferred to naive recipient mice with T lymphocytes obtained from the spleens of *C. neoformans*-sensitized animals, thereby establishing that cell-mediated immunity is responsible for host defense in cryptococcosis.

Sensitized T lymphocytes are known to have the ability to kill or to inhibit the growth of infectious agents (3, 17, 32, 43-46); however, their direct effects on cryptococci have not been investigated.

The primary objectives of the present study were to develop an in vitro assay system for detecting *C. neoformans*-sensitized lymphoid cells in the spleens of mice and to examine more directly their effects on *C. neoformans* by monitoring their interactions in an in vitro system. In addition, this system served as a useful tool to begin characterizing the effector cells.

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MATERIALS AND METHODS

Mice. Inbred CBA/J and A.SW/SnJ mice of both sexes, aged 8 to 12 weeks, were either purchased from Jackson Laboratory, Bar Harbor, Maine, or bred in the animal facilities in the Department of Botany and Microbiology of the University of Oklahoma.

Organism. *Cryptococcus neoformans* isolate 184 A, described by Murphy and Cozad (41), was used for the sensitization of animals and as target cells in the in vitro growth inhibition assays. The cultures were grown on modified Sabouraud agar (MSAB) slants and maintained by biweekly passage on the same medium.

Cryptococcal antigen. The CneF antigen was prepared by the procedures described by Cauley and Murphy (8). This preparation contained 4.0 mg of protein and 4.0 mg of carbohydrate per ml, as determined by the Lowry et al. (36) and phenol-sulfuric acid (15) methods, respectively. Bovine serum albumin was used as a standard, and Monitrol I (Dade Division American Hospital Supply Corp., Miami, Fla.) was used as a control for protein determination. Mannose and mannan served as standard and control, respectively, in the carbohydrate determination.

Sensitization of animals. *C. neoformans* was grown on MSAB agar slants for 3 days, and the cells were harvested and washed three times in sterile physiological saline. The washed cells were heat killed by incubation at 60°C for 60 to 90 min, depending on the number of cryptococci. After heat treatment, viability was determined by plating 0.1 ml of the suspension on MSAB plates. No growth on the plates after 3 to 7 days indicated successful heat treatment. The nonviable cells were washed three times and suspended in sterile physiological saline. Each mouse was given a total of 10^7 heat-killed cryptococci in two 0.05-ml volumes at two sites on the upper abdomen as described by Graybill and Taylor (21). Control mice received an emulsion of sterile physiological saline and complete Freund adjuvant (CFA) in the same fashion or remained unsensitized. Preliminary experiments indicated that splenic cells from these two types of controls responded similarly in the growth inhibition assay.

DTH response. Footpad testing for DTH responsiveness to CneF antigen was performed by the procedures described by Cauley and Murphy (8). A net increase of 0.3 mm or more in footpad swelling was considered a positive response.

Lymphocyte preparation. Spleens and lymph nodes were collected from sensitized and control mice. Single-cell suspensions were prepared by pressing the tissues through a 100-mesh stainless steel screen into sterile Dulbecco phosphate-buffered saline solutions. Erythrocytes were lysed by treating the cell pools with ammonium chloride-Tris-buffered solution (7). The cells were washed three times in Dulbecco saline solution before being suspended in RPMI-1640 culture medium (GIBCO Laboratories, Grand Island, N.Y.) supplemented with 100 U of penicillin per ml, 100 µg of streptomycin per ml, and 10% heat-inactivated fetal calf serum (Flow Laboratories, Inc., Rockville, Md.).

Antisera. Anti-Ia^k, anti-Ia^s, and anti-mouse immunoglobulin (anti-MIg) sera were provided and characterized (39, 40) by John Moorhead, University of Colorado Medical School, Denver, Colo. The anti-Thy-1 serum provided by Paul A. Barstad, Depart-

ment of Botany and Microbiology, University of Oklahoma, was prepared by immunizing goats with a preparation of purified Thy-1 antigen (52), as described by Murphy and Moorhead (42). Dilutions of 1:10 of the anti-Ia^k, anti-Ia^s, anti-MIg, or normal mouse sera and 1:100 of the anti-Thy-1 serum were used along with low-toxicity rabbit serum as a complement (C) source for treatment of splenic and T-enriched lymphocyte populations.

Nylon wool enrichment for T and B lymphocytes. Nylon wool (Leukopak leukocyte filter, Fenwal Laboratories, Deerfield, Ill.) was washed, and the methods of Julius et al. (27) and Handwerger and Schwartz (22) were used for separation and enrichment of T and B cells, respectively. The recovery of nonadherent (T-enriched) cells ranged from 25 to 38% and consisted of 98 to 100% T lymphocytes as determined by trypan blue dye exclusion test after treatment with anti-Thy-1 serum and C, less than 1.5% B lymphocytes as determined by treatment with rabbit anti-MIg and fluorescein-labeled goat anti-rabbit immunoglobulin, and less than 0.8% macrophages based on nonspecific esterase staining (29). Of the cells put on the column, 40% were recovered in the adherent (B-enriched) cell fraction. They consisted of 70 to 84% immunoglobulin-positive cells and 10 to 15% T lymphocytes as determined by methods described above. Viability of both nonadherent and adherent cell populations exceeded 95% as determined by trypan blue dye exclusion.

Antiserum treatment of cells. For treatment of splenic or T-enriched lymphocytes, 10^7 cells were pelleted in tubes (12 by 75 mm; Falcon Plastics, Oxnard, Calif.), 1 ml of the diluted antiserum was added, and the tubes were mixed by gentle shaking. After incubation at 4°C for 1 h, the cells were washed, and 1 ml of a 1:14 dilution of rabbit C was added. The cell suspensions were then incubated for 10 min on ice and for 35 min at 37°C. The cells were washed three times, resuspended to the original 1-ml volume, and the viability was determined.

In vitro growth inhibition assay. Effector cells (splenic, T-, or B-enriched populations) in RPMI-1640 medium supplemented with penicillin and streptomycin and 10% fetal calf serum were incubated together with *C. neoformans* targets in tissue culture tubes (16 by 125 mm; Corning Glass Works, Corning, N.Y.) at 37°C in an atmosphere of 5% CO₂-95% air for 24 h unless otherwise specified. Target cell suspensions were adjusted to 10^5 cells/ml, and 0.1 ml was added to the tissue culture tubes. Effector cells were then added from a stock suspension of 10^7 cells/ml to achieve final effector-to-target (E:T) cell ratios of 100:1, 200:1, 300:1, 400:1, or 500:1. The final volume of each assay tube was adjusted to 1.1 ml with culture medium. Assays with each ratio were performed in duplicate. The controls consisted of target cells (0.1 ml) incubated with 1.0 ml of RPMI-1640 culture medium in the absence of effector cells. After a 24-h incubation, unless otherwise specified, samples were removed from each tube, diluted, and plated in duplicate on MSAB plates. Colony counts were made 3 days after plating. With *C. neoformans* isolate 184, the numbers of cryptococci in the control tubes and tubes containing cryptococci and effector cells from nonimmunized animals increased 20- to 40-fold during the 24-h incubation period; therefore, a final dilution of 1:4,000 routinely provided 50 to 100 colonies per plate. To

obtain similar numbers of colonies per plate from tubes with cryptococci plus sensitized effector cells, a final dilution of 1:200 was used. The effects of lymphoid cells on the target cells were expressed as percent inhibition of growth, which was determined by the following formula: % inhibition of growth = [(number of colonies in control - number of colonies in test)/(number of colonies in control)] $\times$ 100.

Transfer of lymphocytes. On day 8 after injection with heat-killed cryptococci or saline, sensitized and normal splenic and lymph node T-enriched populations were prepared. The method for transfer of lymphoid cells was described previously (32). Briefly, recipient mice were divided into four groups, and each group was injected intravenously with 10^4 viable *C. neoformans*. Two hours after *C. neoformans* injection, 10^8 lymphocytes in 0.1 ml of saline were introduced intravenously. Group 1 mice were given normal T-enriched splenic lymphocytes, group 2 mice received sensitized T-enriched splenic lymphocytes, group 3 mice received normal T-enriched lymph node lymphocytes, and group 4 mice were given sensitized T-enriched lymph node cells. At 20 h after lymphocyte transfers, recipient mice from each group were autopsied, and the numbers of *C. neoformans* colony-forming units in the spleen, liver, lungs, and brain were determined for each mouse by plating dilutions of homogenized tissues on MSAB plates (32).

Statistical analysis. The means, standard errors of the means, and statistical evaluations of the data were analyzed with a Hewlett-Packard calculator model 9810A.

RESULTS

DTH profile and the development of effector cells. At days 2, 4, 6, 8, 16, and 24 after immunization, 4 to 5 mice from sensitized or control groups were footpad tested with CneF antigen to determine DTH responsiveness. Mice from control groups did not show increased footpad swelling in response to CneF antigen; however, mice sensitized with the heat-killed cryptococci-CFA emulsion began showing positive DTH responses by day 6 after immunization. The intensity of the responses increased significantly, reaching a maximum between 8 and 16 days after immunization (Fig. 1A).

Kinetic studies on the development of effector cells that inhibited the growth of *C. neoformans* were performed, using the in vitro inhibition assay. Splenic cells for this assay were collected at the same times after immunization that DTH responses were measured. E:T cell ratios of 100:1, 200:1, 300:1, 400:1, and 500:1 were used in the experiments, and the cell suspensions were incubated at 37°C for 24 h. The results are shown in Fig. 1B. Effector cells from control mice did not inhibit the growth of *C. neoformans* at any E:T ratio tested; however, effector cells from mice sensitized 6 days previously with cryptococci-CFA were inhibitory to the cryptococci at E:T ratios of 300:1 or greater. By day 8 after immunization, the growth-inhibitory activi-

ty attained a maximal level and continued to be high through day 24. The highest inhibition of growth was observed at E:T ratios above 300:1, whereas there was no detectable activity at E:T ratios of less than 300:1. For convenience, in further experiments effector cells were collected on day 8 after immunization, and an E:T ratio of 300:1 was used unless otherwise specified.

Effect of T- and B-enriched lymphocytes on *C. neoformans*. For a determination of whether the effector cells which inhibited the growth of *C. neoformans* were T or B cells, sensitized splenic lymphocytes collected on day 8 after immunization were separated on a nylon wool column into adherent (B-enriched) and nonadherent (T-enriched) pools and tested for growth-inhibitory ability on the pathogen. Effects of immune B-enriched cells on *C. neoformans* were determined at E:T ratios of 100:1 and 300:1, and effects of unfractionated immune splenic and T-enriched cells were determined at E:T ratios of 100:1 through 500:1. Unfractionated *C. neoformans*-sensitized splenic cells were able to inhibit the growth of *C. neoformans* at E:T ratios of 300:1, 400:1, and 500:1 (Fig. 2), whereas the sensitized T-enriched populations inhibited the growth of the pathogen at all E:T ratios examined. The nylon wool adherent (B-enriched) populations were unable to inhibit the cryptococci at an E:T ratio of 100:1 and exhibited a significantly lower ability to inhibit *C. neoformans* at an E:T ratio of 300:1 than did the unfractionated splenic or T-enriched effector cell populations ($P < 0.01$). The percentage of inhibition exhibited by the T-enriched lymphocytes was considerably higher ($P < 0.01$) than that seen with the unfractionated splenic population at E:T ratios of 100:1 and 200:1, but at other E:T ratios, T-enriched cells exerted only slightly higher levels of growth inhibition on the pathogen. Effector cells from normal mice did not exhibit inhibition of growth of *C. neoformans* at any of the E:T ratios examined.

Kinetics of growth inhibition. Inhibition of growth of *C. neoformans* by sensitized effector cells was analyzed at various times after effector and target cells were mixed. Growth inhibition patterns exhibited by sensitized splenic and T-enriched cell populations were identical in that inhibition of growth was observed as early as 4 h and increased gradually, peaking at 24 h. By 48 h, the ability to inhibit the growth of the target cells had dropped considerably. Control cell populations showed no effect on *C. neoformans* at any time. The growth-inhibitory ability of sensitized T-enriched cells was greater than that of unfractionated splenic cells from the same animals, and the inhibitory activities of the two-cell populations were significantly different ($P < 0.05$) after 24 h of incubation, the time when

FIG. 1. (A) Mean increase in footpad thickness 24 h after footpad testing with a cryptococcal culture filtrate. Vertical bars designate standard errors of the means. Symbols: ▲, mice immunized with heat-killed *C. neoformans*-CFA emulsion; ○, mice immunized with sterile saline-CFA emulsion. (B) Profile of the development of effector splenic cells which were capable of inhibiting the growth of *C. neoformans* in vitro. Symbols for sensitized lymphocytes: ▲, E:T ratio of 300:1; ■, E:T ratio of 400:1; ★, E:T ratio of 500:1; ○, E:T ratios of 100:1 and 200:1. Symbols for normal lymphocytes: ○, E:T ratios of 100:1 to 500:1.

both effector cell populations displayed maximum effects on the organism (Fig. 3). In similar experiments, we consistently noted that sensitized T-enriched populations were more effective in inhibiting *C. neoformans* than were sensitized splenic nonseparated populations; however, occasionally those differences were not statistically significant.

Supplement with medium and lymphoid cells. In all experiments performed in this study, the maximum inhibition of growth of *C. neoformans* occurred at 24 h, but by 48 h the growth-inhibitory activities had substantially waned. The loss of inhibitory activity could have been

due either to death of effector cells or to the loss of cellular function resulting from the depletion of essential nutrients. To determine whether the inhibitory ability could be maintained or restored beyond 24 h, we added equal volumes of fresh medium or an additional 3×10^6 sensitized effector cells to the reaction mixtures at 12 and 24 h, and allowed the mixtures to incubate up to 48 h. Inhibition of growth of *C. neoformans* was enhanced significantly at 48 h in those groups that received supplements of either fresh medium or effector cells (Table 1). A twofold or greater inhibition of growth at 48 h was observed in those groups receiving either supplement.

FIG. 2. Comparison of the ability of sensitized splenic, B- and T-enriched lymphocytes to inhibit the growth of *C. neoformans* at various E:T ratios. Effector lymphoid cells were collected from mice on day 8 after immunization and incubated with target cells at 37°C for 24 h. Vertical bars represent standard errors of the means. Symbols: ▲, *C. neoformans*-sensitized unfractionated splenic lymphocytes; △, *C. neoformans*-sensitized splenic T-enriched lymphocytes; □, *C. neoformans*-sensitized splenic B-enriched lymphocytes; ●, normal unfractionated splenic lymphocytes; ○, normal splenic T-enriched lymphocytes.

Further characterization of effector cells. Earlier experiments in this study strongly indicated that the nylon wool nonadherent cells were the major effector cells responsible for the inhibition of growth of *C. neoformans*. This finding was further substantiated by the observations that treatment of the nylon wool nonadherent effector cells with anti-Thy-1 serum plus C abolished the ability to inhibit the growth of cryptococci (Fig. 4). On the other hand, pretreatment of effector splenic or T-enriched cells with a 1:10 dilution of normal mouse or anti-MIg sera and C or C alone did not affect the percentage of growth inhibition.

Effector T lymphocytes were further characterized by anti-Ia serum plus C treatments. Anti-Ia^k and anti-Ia^s sera specific for Ia-bearing cells of CBA/J and A.SW mice, respectively, were used. CBA/J and A.SW mice were first immunized with heat-killed *C. neoformans*-CFA emulsion. Eight days after immunization, T-enriched lymphocytes were collected, and portions were treated with anti-Ia^k or anti-Ia^s serum plus C. Antisera and C-treated and untreated T-enriched lymphocytes were assessed for their ability to inhibit *C. neoformans* growth (Fig. 5). Results revealed that treatment of CBA/J effector T-enriched lymphocytes with anti-Ia^k plus C completely abolished their growth-inhibitory abilities, whereas the CBA/J effector cells were

unaffected by treatment with anti-Ia^s serum and C. Similar results were observed when A.SW T-enriched splenic lymphocytes were treated; anti-Ia^s plus C abolished the activity, and anti-Ia^k plus C had no effect. Results obtained in this experiment provided evidence that the effector T lymphocytes belong to an Ia⁺ subpopulation.

Comparison of inhibition of growth using in vitro and in vivo systems. An experiment was conducted to correlate the in vitro growth inhibition with in vivo inhibition of *C. neoformans* as described by Lim and Murphy (32). Normal and sensitized T-enriched lymphocyte pools were divided into two portions. One portion of each pool was used in the in vitro assay to assess the percentage of *C. neoformans* growth inhibition, and the other portion was transferred to recipient mice which had been given an intravenous challenge with viable *C. neoformans* 2 h earlier. At 20 h after challenge, the total numbers of *C. neoformans* colony-forming units in various organs of the recipients were determined. The results are shown in Fig. 6. In the in vitro assay, sensitized T cells inhibited the growth of *C. neoformans* by 60 to 80%, whereas normal T-enriched cells inhibited the growth by only 20 to 35%. In the in vivo system, animals given normal T lymphocytes had approximately five to twenty times as many culturable *C. neoformans* as did animals given sensitized T lymphocytes.

FIG. 3. Kinetic studies of the growth-inhibiting effects of immune splenic and T-enriched lymphocytes on *C. neoformans* as targets. Effector cells, collected 8 days after immunization of mice with heat-killed cryptococci or saline, were incubated with *C. neoformans* at an E:T ratio of 300:1 at 37°C for various periods of time. Symbols: Δ, immune T-enriched lymphocytes; ▲, immune splenic lymphocytes; ●, normal T-enriched lymphocytes; ○, normal splenic lymphocytes.

TABLE 1. Effect of the addition of fresh medium or fresh immune lymphoid cells on the inhibition of growth of *C. neoformans*^a

Cell population	Supplement at 12 and 24 h	% Inhibition at 24 h ^b	% Inhibition at 48 h
Immune spleen	None	86	36.3
	Fresh medium	ND	70.0
	Fresh immune cells	ND	88.5
Immune splenic T-enriched	None	83.7	33.2
	Fresh medium	ND	64.8
	Fresh immune cells	ND	75.6

^a On day 8 after immunization, splenic and T-enriched lymphocytes were collected from *C. neoformans*-sensitized animals and were incubated with *C. neoformans* at an E:T ratio of 300:1 at 37°C. After 12 and 24 h of incubation, equal volumes of fresh medium, 3×10^6 fresh splenic or T-enriched lymphocytes were added to the sensitized splenic-*C. neoformans* and the sensitized T-enriched-*C. neoformans* reaction mixtures, respectively. All reaction mixtures were allowed to incubate up to 48 h.

^b ND, Not done.

The inhibition of growth of *C. neoformans* by sensitized T lymphocytes in vitro coincided with the reduced numbers of culturable cryptococci from mice that received sensitized T lymphocytes.

In this experiment (Fig. 6) and several others done in our laboratory (data not shown), we have observed that splenic and splenic T-enriched cell populations from normal CBA/J mice inhibited the growth of *C. neoformans* in vitro. The *C. neoformans* growth-inhibitory activity of normal splenic cell populations varies with the age of donor mice and is most likely due to the presence of natural killer cells (41a). The donor mice used in generating the data shown in Fig. 6 were 8-week-old animals; therefore, their natural killer reactivity was still at a measurable level. In other experiments presented here, the animals used were approximately 11 to 12 weeks of age, an age when splenic natural killer activity is relatively low; therefore, the normal splenic cells did not inhibit the growth of *C. neoformans* (Fig. 1B, 2, and 3). The data presented in Fig. 6 clearly indicate that the cryptococci growth-inhibitory activity was greatly enhanced by immunization, and the data in Fig. 2 and 4 show that the effector cells are T cells, thereby eliminating the possibility that augmentation of natural killer cell reactivity was responsible for the enhanced activity seen in the sensitized mice.

DISCUSSION

An in vitro growth inhibition assay was developed which not only allowed a direct examination of the effect of sensitized lymphoid cells on

FIG. 4. Effects of anti-Thy-1 or anti-MIg serum and rabbit complement (C) treatment on the ability of sensitized splenic and T-enriched cells to inhibit the growth of *C. neoformans*. Effector cells were collected on day 8 after immunization. E:T ratio was 300:1. Incubation time was 24 h at 37°C. Bars: ▨, immune splenic lymphocytes; □, immune splenic T-enriched lymphocytes.

TREATMENT	% <i>C. neoformans</i> GROWTH INHIBITION																	
	SENSITIZED CBA/J SPLENIC CELLS									SENSITIZED A.S.W SPLENIC CELLS								
	10	20	30	40	50	60	70	80		10	20	30	40	50	60	70	80	90
UNTREATED																		
C CONTROL																		
NMS + C										ND								
ANTI-Ia ^k + C																		
ANTI-Ia ^s + C																		

FIG. 5. Effect of normal mouse serum (NMS), anti-Ia^k serum, and anti-Ia^s serum, plus complement (C) on the ability of sensitized effector T-enriched lymphocytes to inhibit the growth of *C. neoformans*. Effector T lymphocytes were prepared from mice on day 8 after immunization. E:T ratio was 300:1, and incubation time was 24 h at 37°C. Vertical bars represent standard errors of the means. ND, Not done.

C. neoformans but also provided a tool for further investigations on the kinetics of interactions between the effector cells and the pathogen and for characterization of the effector cells. In addition, this work reaffirmed the importance of cell-mediated immunity, particularly the T-lymphocyte function in the host resistance to cryptococcosis.

In this study, lymphoid cells from *C. neoformans*-sensitized animals were shown to inhibit the growth of *C. neoformans* in an in vitro

growth inhibition assay. The inhibitory cells developed coincidentally with the increase of DTH responsiveness of the mice. Parallel developments of DTH responsiveness and effector cell functions, such as the resistance to further challenge with *C. neoformans* (20, 21, 32), the restriction of proliferation of viable cryptococci in various tissues (8, 32, 33), and the production of macrophage migration inhibition factor (23, 33) have also been demonstrated in other host-*C. neoformans* studies. It has been shown with

FIG. 6. Comparison of the inhibition of growth of *C. neoformans* in vitro and in vivo (lymphocyte transfer) assays. In the in vitro assay, normal and sensitized splenic or lymph node T-enriched lymphocytes obtained on day 8 after immunization were incubated with *C. neoformans* at an E:T ratio of 300:1 for 24 h at 37°C. The same T-enriched lymphocyte populations (10^8 cells) were transferred to groups of recipient mice which had been given 10^4 viable *C. neoformans* 2 h before lymphoid cell transfers. Numbers of viable *C. neoformans* (colony-forming units) from the spleens, livers, lungs, and brains of each mouse were determined and summed 20 h after lymphoid cell transfer. Vertical bars represent the standard errors of the means.

other models that DTH activities and cellular immune functions, such as cytotoxic responses to sheep erythrocytes or lymphocyte-proliferative responses, were mediated by different T-lymphocyte subclasses (25, 39). Whether the DTH responses and growth inhibition activities described in this study were manifested by the same or different T-cell subpopulations was not established. Nevertheless, these findings provided further evidence that DTH responsiveness and effective immunity are coexisting features in murine cryptococcosis.

With the in vitro growth inhibition assay, we were able to provide direct evidence that T cells, but not B cells or macrophages, were the major effector cells responsible for inhibiting the growth of *C. neoformans*. Two approaches were used to demonstrate this. First, T and B lymphocytes were enriched on nylon wool columns and tested for growth-inhibitory activities against *C. neoformans* in the in vitro assay. T-enriched lymphocytes were able to inhibit the growth of cryptococci at lower E:T ratios (e.g., 100:1 and 200:1) than were the sensitized unfractionated splenic cells (Fig. 2–4). Although the B-enriched cell populations demonstrated a slight ability to inhibit *Cryptococcus* growth at an E:T ratio of 300:1, the inhibition was considerably less than that observed with the unfractionated splenic or T-enriched populations. The inhibitory ability of B-enriched fractions could have been due to the fact that 10 to 15% of the cells in those fractions were Thy-1-positive cells or T cells. Although macrophages have been shown to be capable of engulfing *C. neoformans* in human or animal cryptococcosis (5, 6, 37), they were not likely to have played a role in this system since the depletion of macrophages from *C. neoformans*-

sensitized splenic cell pools did not affect the ability of the cells to inhibit the growth of the pathogen. Second, the effector cells were shown to be T lymphocytes by the fact that anti-Thy-1 serum plus C abolished the inhibitory ability, and anti-MIg and C did not affect the activity. These results supported the findings of Lim and Murphy (32), who demonstrated that sensitized T-enriched cells adoptively transferred resistance to a cryptococcal infection and that the resistance could be abolished by treating the effector cells with anti-Thy-1.2 serum and C before adoptive transfer.

Recently, Monga et al. (38) showed that B cells were not involved in resistance in murine cryptococcosis by demonstrating that there were no differences between B-cell-deficient and normal control animals with respect to their mortality patterns, viable cryptococci counts in different organs, DTH responses to CneF antigen, or antigen levels in the sera. They concluded that cells other than B cells or B-cell products had to be responsible for the protection or elimination of the cryptococci.

One of our objectives in this study was to determine whether the effector lymphoid population which was capable of inhibiting *C. neoformans* growth in vitro could also perform in vivo by restricting the proliferation of *C. neoformans*, as reported by Lim and Murphy recently (32). Results (Fig. 6) in this study demonstrated that *C. neoformans*-sensitized T-enriched splenic as well as lymph node lymphocytes not only inhibited the growth of the pathogen in vitro but also restricted *C. neoformans* proliferation in various vital organs when adoptively transferred to naive *C. neoformans*-challenged recipients. These results added support to the concept that sensi-

tized T lymphocytes play a major role in protection against cryptococcal infection.

The kinetics of growth inhibition exhibited by both sensitized splenic and T-enriched lymphocytes were very similar. They exhibited growth-inhibitory activities as early as 4 h after effector and target cells were mixed, and the activities increased gradually, peaking at 24 h. By 48 h, the activity in both effector groups had dropped significantly. This drop could have been due to death of effector cells or to the loss of cellular functions resulting from the depletion of essential nutrients. We demonstrated that the growth-inhibitory activities of the sensitized effector cells could be restored with the addition of either fresh medium or sensitized effector cells, indicating that either or both of these situations were potential explanations for the drop in activity. In an *in vivo* situation, since there is a constant and plentiful supply of fresh nutrients and sensitized effector cells circulating to the focal point of infection, we postulate that the proliferation of *C. neoformans* would be restricted effectively by the sensitized cells.

Characterization of the effector cells was made possible by the *in vitro* growth inhibition assay. Results revealed that the effector T lymphocytes were Ia^+ , as determined by the fact that their growth-inhibitory abilities could be completely abolished upon treatment with anti- Ia^k or anti- Ia^s serum and C in CBA/J or A.SW mice, respectively. It has been reported that T lymphocytes responsible for the DTH response, the T_{DH} cells, in the 2,4-dinitrofluorobenzene system were Ia^- T cells (39). Similar findings have been reported in other systems (34, 48). In contrast to the T_{DH} cells, cytotoxic T lymphocytes (T_C) generated in the mixed-lymphocyte culture reactions resembled the T_{DH} cells in that they were both nylon wool nonadherent and had Thy-1 on their surfaces, but T_C cells differed from T_{DH} cells in that they have been reported to be unable to transfer DTH responsiveness to recipient animals, and in some cases, T_C cells have been shown to have Ia antigens on their surfaces (18, 28; N. R. Pellis, C. M. Macek, and B. D. Kahan, Fed. Proc. 40:1118, 1981). In this study, the effector T (T_{INH}) lymphocytes capable of inhibiting the growth of *C. neoformans* were shown to be nylon wool nonadherent, Thy-1⁺, and Ia^+ , characteristics which more closely resemble T_C cells than T_{DH} cells in other models. Further work must be done to establish the similarities and differences between T_{INH} cells in this system and T cells responsible for transfer of DTH responsiveness.

Recently, Yu et al. (50) reported that the percentage of Ia^+ T lymphocytes was elevated in the circulation of patients with various diseases such as rheumatoid arthritis, infectious

mononucleosis, or graft-versus-host diseases. Elevation of Ia^+ T lymphocytes also occurred in individuals after stimulation with antigens such as tetanus toxoid, purified protein derivative (50), or mitogens (31, 50). It has also been reported that Ia^+ T cells in large numbers appeared approximately 7 days after stimulation with pokeweed mitogen (50). The Ia^+ cells in this study which were responsible for the inhibitory activity on *C. neoformans* may be cells similar to the ones mentioned above. During a cryptococcal infection there may be an induction of Ia^+ T lymphocytes which could specifically inhibit the growth of *C. neoformans* during the course of cryptococcosis, accounting for the protection of the host against the infection. Further studies must be done to establish whether Ia^+ T lymphocytes can be found in increased numbers in circulation during cryptococcosis.

Cell-mediated immunity is probably the most important defense mechanism, not only in cryptococcosis but also in other systemic fungal infections such as coccidioidomycosis, blastomycosis, and histoplasmosis (2, 3, 49), and the effector cells responsible for host resistance to these pathogens have not been fully characterized. The *in vitro* growth inhibition assay developed in the present study may provide a tool for further characterization of the effector cells in cryptococcosis as well as in other mycoses. This technique may also have potential in defining the level of resistance of a host to specific organisms and in establishing a better understanding of the mechanisms of inhibition of the pathogen.

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