Direct Anticryptococcal Activity of Lymphocytes from *Cryptococcus neoformans*-Immunized Mice

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Assessment of the direct anticryptococcal activity of murine lymphocytes from both *Cryptococcus neoformans*-immunized and control mice was the focus of this investigation. We demonstrate that at a 2:1 effector cell-to-cryptococcal target cell ratio, effector cell populations comprised of $\alpha\beta$ T-cell receptor-positive T lymphocytes (98 to 99% CD3⁺) from *C. neoformans*-immunized mice inhibited the growth of cryptococcal cells better than similar populations of lymphocytes from nonimmunized control mice. Almost immediately after mixing of cryptococci with the effector cells, *C. neoformans*-lymphocyte conjugates were observed. The percentage of conjugates increased over the first 30 min of incubation and then remained constant over the next 1.5 h. T-lymphocyte-enriched populations from *C. neoformans*-immunized mice formed significantly greater percentages of conjugates with cryptococci than control T lymphocytes at each time period that assessment was made. For growth inhibition to occur, direct contact between the effector and target cells was necessary, as evidenced by abrogation of cryptococcal growth inhibition when lymphocyte and cryptococcal cell populations were separated by a porous membrane during the growth inhibition assay. Vital staining of cryptococci after incubation with the T-cell-enriched populations showed that the T lymphocytes killed the cryptococcal cells.

The incidence of the opportunistic fungal disease cryptococcosis has been rising over recent years due, in large part, to an increasing immunocompromised population resulting from immunosuppressive therapies and AIDS (4, 22, 42). Systemic infections with *Cryptococcus neoformans* are frequently fatal for patients with compromised cell-mediated immune (CMI) responses (5, 10) even when current therapies are administered (9, 29, 39). At the present time, the host mechanisms by which *C. neoformans* infections are cleared from the immunocompetent host are not thoroughly understood. It is evident, however, from previous studies that T lymphocytes are important in protection against *C. neoformans* (15, 16, 27, 30).

There are two means by which T lymphocytes in a CMI response can aid in the clearance of infecting microorganisms. The most widely studied means is through the indirect actions of sensitized or memory T lymphocytes (28). In this case, immune T cells, upon reexposure to the inducing antigen, produce lymphokines which activate natural effector cells such as neutrophils, monocyte/macrophages, and natural killer (NK) cells to more effectively kill the invading organism. There is some evidence that such a mechanism may play a role in the elimination of *C. neoformans* from the host (1, 8, 26). The second and less studied means by which T lymphocytes might affect the clearance of an invading microbe is through direct interactions with the organism to mediate the death of the organism or to limit multiplication of the organism. Most studies involving the direct mechanism of anticryptococcal growth inhibition have been done with murine natural killer (NK) cells (14, 33, 34, 36). However, there is one report of a study in which anticryptococcal activity was demonstrated in vitro with Thy-1⁺, nylon wool-nonadherent (NWN) splenocytes and NWN lymph node cells from mice immunized with *C. neoformans* but was lacking or greatly reduced in similar cell populations from saline-injected control mice (7). More recently,

freshly isolated human T lymphocytes (CD3⁺ cells) have been shown to bind to *C. neoformans* (33). Furthermore, freshly isolated human lymphocyte populations highly enriched for T cells inhibit the growth of cryptococcal cells (25, 33). The studies with human lymphocytes did not address, however, whether or not the individuals from whom the lymphocytes were obtained were sensitized to some degree to *C. neoformans*. Considering that *C. neoformans* is a ubiquitous organism, it is possible that some, if not all, of the individuals have encountered cryptococci previously and could have become sensitized. A murine model can be used to determine the effects of *C. neoformans* exposure on the ability of the T lymphocytes to display direct anticryptococcal activity.

The purposes of this study were to compare murine CD3⁺ lymphocyte-enriched populations from *C. neoformans*-immunized mice with similar T-cell populations from saline-treated control mice for the ability to mediate growth inhibition of *C. neoformans* by using a microassay procedure and to determine if the anticryptococcal activity required direct interactions of the lymphocytes with the cryptococcal cells. First, we confirmed that splenic and lymph node cell populations highly enriched for T lymphocytes (CD3⁺ cells) obtained from *C. neoformans*-immunized mice inhibit the growth of cryptococcal cells better than similarly isolated T lymphocytes from nonimmunized control mice. Second, we found that a greater percentage of T lymphocytes from *C. neoformans*-immunized mice form conjugates with cryptococcal cells than do lymphocytes from control mice. Third, we provide evidence that cell-to-cell contact between the T lymphocytes and the cryptococcal cells is required for inhibition of cryptococcal growth. Finally, by assessing the viability of cryptococci with an assay for metabolic activity, we found that the inhibition in *C. neoformans* proliferation is due to death of the cryptococcal target cells.

MATERIALS AND METHODS

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Mice. Inbred, female CBA/J mice (*H-2^k*) were purchased from Jackson Laboratory, Bar Harbor, Maine. The animals were maintained in the University of

Oklahoma Health Science Center animal facility and used between 8 and 12 weeks of age.

Organisms. *C. neoformans* isolate 184-A was maintained on modified Sabouraud agar (MSAB) slants (31). Heat-killed *C. neoformans* for immunization was prepared by harvesting the blastoconidia in endotoxin-free sterile physiologic saline solution (SPSS) from the MSAB slants after 3 days of growth at room temperature and then incubating the cryptococci for 1 h at 60°C. After heat treatment, the sterility of the culture was demonstrated by the lack of growth on MSAB plates. The mean capsule width $\pm$ 1 standard deviation for isolate 184A cells grown on MSAB was 0.58 ± 0.19 μ m.

Immunization with *C. neoformans*. An anticytotoxic CMI response was induced in mice as previously described (35). Briefly, mice were injected with 0.1 ml of SPSS containing 10^7 heat-killed *C. neoformans* cells subcutaneously at two sites on the abdomen of the mice. Control mice were similarly injected with an equal volume of SPSS. Eight days after the injections, spleens and lymph nodes were collected and single-cell suspensions were made. The efficacy of the immunization was demonstrated in a separate group of mice simultaneously treated and then footpad tested with cryptococcal culture filtrate antigen (CneF) (3). In all cases, the mice immunized with heat-killed *C. neoformans* displayed anticytotoxic delayed-type hypersensitivity reactions whereas control mice did not (data not shown), indicating that the immunized mice had developed an anticytotoxic CMI response.

Preparation of effector and target cell populations. Spleen cell suspensions (SPC) composed of spleens from at least three mice or popliteal, inguinal, and mesenteric lymph node cell suspensions (LN) composed of nodes from at least five mice were passed over nylon wool columns (17). The NWN cells were collected and used as effector cells in the conjugation and cryptococcal growth inhibition assays. All effector cell populations were suspended in tissue culture media (TCM) consisting of RPMI 1640 with 10% fetal bovine serum. The target cells for the conjugation and growth inhibition assays were single-cell suspensions of *C. neoformans* cells. For target cell preparations, *C. neoformans* isolate 184A was grown on MSAB for 1 day at room temperature, then transferred to 10 ml of TCM, and grown overnight at 37°C in 7% CO₂. The cryptococcal cells grown in TCM were washed three times and then resuspended in fresh TCM before being used in the assays. The mean capsule width $\pm$ 1 standard deviation for isolate 184A cells after incubation in TCM and 7% CO₂ was 1.3 ± 0.45 μ m.

Antibodies. The immunoglobulins used for direct fluorescent labeling of cells in the flow cytometric analyses of surface markers were purchased from Pharmingen (San Diego, Calif.) unless otherwise indicated. They included fluorescein isothiocyanate-conjugated hamster immunoglobulin G (IgG) anti-mouse CD3, hamster IgG anti-mouse $\alpha\beta$ T-cell receptor (TCR), hamster IgG anti-mouse $\gamma\delta$ TCR, purified hamster IgG as a control, rat IgG2b anti-mouse Lyt 2 (CD8), and rat IgG2b (an isotype control), as well as phycoerythrin-conjugated rat IgG2b anti-mouse L3/T4 (CD4) (CALTAG Laboratories, San Francisco, Calif.) and phycoerythrin-conjugated rat IgG2b (an isotype control). Primary polyclonal antibodies used in indirect fluorescent labeling of cells were purified rabbit anti-mouse IgM μ chain (Jackson ImmunoResearch Laboratories, West Grove, Pa.) and rabbit anti-mouse asialo-GM-1 (Wako Chemicals USA, Dallas, Tex.). The secondary antibody used with the polyclonal rabbit antibodies was fluorescein isothiocyanate-conjugated goat anti-rabbit IgG (CALTAG).

Phenotyping of effector cells. Flow cytometric analysis was used to define the phenotypes of the effector cell populations. For this, 10^6 SPC, LN, NWN-SPC, or NWN-LN cells were subjected to either a direct or indirect immunofluorescent labeling procedure. Direct labeling was performed by incubating cell samples with 1 μ g of fluorescently labeled immunoglobulin diluted in 0.1 ml Hanks' balanced salts solution with 1% bovine serum albumin (BSA) and 0.1% Na₂S₂O₃ for 30 min. Indirect immunofluorescent labeling was achieved by incubating cell suspensions with 1 μ g of the primary antibody in 0.1 ml of Hanks' balanced salts solution with 1% BSA and 0.1% Na₂S₂O₃ for 30 min; then after washing, the cells were incubated with 1 μ g of fluorescently labeled secondary immunoglobulin in 0.1 ml of Hanks' balanced salts solution with 1% BSA and 0.1% Na₂S₂O₃ for 30 min. The labeled cells were washed and fixed in phosphate-buffered saline with 0.1% Na₂S₂O₃ and 1% paraformaldehyde. Stained cells were analyzed on a FACStar^{PLUS} scanner (Becton Dickinson). The percentage of macrophages in the populations was determined in nonspecific esterase-stained preparations (21).

In vivo depletion of asialo-GM-1⁺ cells. On day -1, mice were given intravenously 0.1 ml of a 1/10 dilution of normal rabbit serum as a control or the same volume of a 1/10 dilution of rabbit anti-asialo-GM-1 antibody to deplete mice of NK cells and cytotoxic T cells. On day 0, the mice were treated with SPSS or SPSS containing 10^7 heat-killed *C. neoformans* cells as described above. The SPC were obtained on day 8 and subjected to purification procedures. The NWN-SPC were subjected to flow cytometric analysis as described above for phenotyping, to determinations of NK activity with a standard assay for ⁵¹Cr release from YAC-1 target cells (34), and to the *C. neoformans* growth inhibition assay.

Assay for growth inhibition of *C. neoformans*. The growth inhibition assay previously used by Fung and Murphy (7) to assess T-lymphocyte-mediated growth inhibition of *C. neoformans* was replaced with a procedure used by Hidore et al. (14) for assessment of murine NK cell activity against *C. neoformans*. Briefly, quadruplicate test wells were prepared in flat-bottom 96-well microtiter plates (Limbro Scientific Co., Hamden, N.Y.). Each test well contained 10^6 effector cells and 5×10^5 viable *C. neoformans* cells in 0.2 ml of TCM. For controls, 5×10^5 viable *C. neoformans* cells in 0.2 ml of TCM were added to

quadruplicate wells. The assay mixtures were incubated at 37°C for 24 h in 7% CO₂. After the incubation, cells in the wells were mixed and samples were removed, diluted, and plated on MSAB plates. The plates were incubated for 3 days at room temperature, and the CFU were counted. The percent growth inhibition was calculated as follows: [(number of CFU from control wells - number of CFU from test wells)/number of CFU from control wells] $\times$ 100.

To determine if direct contact between effector and target cells was required for cryptococcal growth inhibition, some experiments were performed in 24-well, tissue culture-treated Transwell plates (Costar, Cambridge, Mass.). Wells of a Transwell plate have two chambers separated by a 10- μ m polycarbonate membrane having a 0.45- μ m pore size. In the Transwell plates, NWN-SPC ($6 \times 10^6/0.4$ ml of TCM) were added to the upper chamber and viable cryptococcal cells ($3 \times 10^6/0.4$ ml of TCM) were added to lower chamber. Additional test wells contained identical numbers of NWN-SPC and cryptococcal cells but did not have the effector cells and the cryptococcal cells separated by the membrane insert, whereas control wells contained 3×10^6 cryptococcal cells in 0.8 ml of TCM. The total volume in each well was 0.8 ml of TCM. The Transwell plates were incubated at 37°C for 24 h in 7% CO₂, after which time samples of cryptococcal cell suspensions were removed from the bottom chambers, diluted, and plated on MSAB plates. Cryptococcal growth inhibition was determined as described above.

Assay for effector cell-*C. neoformans* conjugate formation. Conjugate formation between effector cells and *C. neoformans* target cells was performed as previously described (32, 36). Briefly, 10^6 effector cells and 5×10^5 cryptococcal target cells suspended in TCM were combined in a total volume of 0.2 ml. The cell mixtures were incubated for the designated time (0 to 2 h) at 37°C in 7% CO₂ before the conjugates were enumerated microscopically to determine the percentage of effector cells attached to *C. neoformans* cells. The effector cell-*C. neoformans* conjugates in a minimum of 200 effector cells were counted for each sample.

MTT vital staining of *C. neoformans* cells. The viability of *C. neoformans* cells after incubation with lymphocytes was assessed with the tetrazolium dye 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) as previously described (14). Briefly, the same numbers of effector and cryptococcal cells used in the growth inhibition and conjugation assays (10^6 lymphocytes and 5×10^5 *C. neoformans* cells) were mixed in wells and incubated for 24 h. The cell mixtures were suspended in 1 ml of serum-free RPMI 1640 containing 0.5 mg MTT per ml and were incubated for an additional 3 h at 37°C in 7% CO₂. The cells were then washed in serum-free RPMI 1640 before being analyzed by light microscopy for the presence of MTT-formazan crystals within viable cells. For determination of the percent viable cryptococci in the total population, the number of cryptococci containing MTT-formazan crystals (live cryptococcal cells) in a minimum of 200 cryptococci (including lymphocyte-bound and unbound) were counted. To determine the percent viable cryptococci bound by lymphocytes, the numbers of cryptococci with MTT-formazan crystals (live cryptococcal cells) in 100 lymphocyte-*C. neoformans* conjugates were counted. The percentage of total killed cryptococci was calculated as [(mean percent viable cryptococci in four control wells - percent viable cryptococci in each experimental well)/mean percent viable cryptococci in four control wells] $\times$ 100. The percentage of killed cryptococcal cells in conjugates was calculated as [(number of conjugates - number of conjugates containing viable cryptococci)/number of conjugates] $\times$ 100.

Statistical analysis. Calculations of means, standard errors of the means, and unpaired Student's *t* test were used to analyze the data. Data are reported with the standard errors of the means. In comparing two groups, *P* values equal to or less than 0.05 were considered to indicate a significant difference between the groups.

RESULTS

Growth inhibition of *C. neoformans* mediated by lymphocytes from immune and control mice. Previous studies have shown that lymphocytes from *C. neoformans*-immunized mice mixed with *C. neoformans* cells in 1.1-ml volumes of TCM at effector/target cell (E/T) ratios of 100:1 to 500:1 inhibit the growth of the organism better than lymphocytes from control mice under the same conditions (7). In the present study to assess the anticytotoxic activity of murine T lymphocytes, we used the microassay protocol of Hidore et al. (14) for measuring direct anticytotoxic activity of murine NK cells. The microassay procedure had several advantages over the procedure used by Fung and Murphy (7) in that the microassay was performed in 96-well microtiter plates rather than tubes and required fewer effector cells and smaller volumes of reagents per assay. Initial experiments were performed to establish that the anticytotoxic activity associated with the T-lymphocyte-enriched fractions of spleen and lymph node cell populations from immunized mice could be detected with the

FIG. 1. Mean percent growth inhibition of *C. neoformans* mediated by designated effector cell populations isolated from SPSS or *C. neoformans*-treated mice. Error bars represent the standard errors of the means. *P* values from unpaired Student *t* tests for comparison of significance between the activities of effector cell populations isolated from SPSS- and from *C. neoformans*-treated mice are in parentheses. Data are representative of three experiments.

microassay at an E/T ratio of 2:1 and in a total volume of 0.2 ml of medium. The anticryptococcal activities mediated by SPC, NWN-SPC, LN, and NWN-LN cell populations from SPSS- and *C. neoformans*-treated mice assessed with the microassay are shown in Fig. 1. Our results are similar to those of Fung and Murphy (7), in that splenic and lymph node effector cells from *C. neoformans*-immunized mice had significantly higher levels of anticryptococcal activity than did comparable effector cell populations from SPSS-treated mice (Fig. 1; $P \leq 0.01$). However, in contrast to the negative results with T-lymphocyte-enriched cell populations from 8- to 12-week-old mice treated with SPSS reported by Fung and Murphy (7), we were able to measure with the microassay low levels of anticryptococcal activity with all of the cell populations from SPSS-treated control mice (Fig. 1). When the SPC or LN cell populations were enriched for T cells and depleted of B cells and macrophages by passage over nylon wool columns, the anticryptococcal activity was enhanced (Fig. 1). This was true irrespective of whether the effector cells were from SPSS- or *C. neoformans*-treated mice (Fig. 1; enrichment of cells from SPSS-treated mice, $P \leq 0.005$; enrichment of cells from *C. neoformans*-treated mice, $P \leq 0.01$). The inhibition of cryptococcal growth in the microassay was not the result of nutrient depletion of the medium by effector cells during the assay, because cryptococci grew equally well or better in spent medium obtained from strongly positive growth inhibition assays ($[1.5 \pm 0.2] \times 10^6$ cryptococci per well) than they grew in fresh TCM ($[1.2 \pm 0.1] \times 10^6$ cryptococci per well).

Phenotyping of effector cell populations. The enhanced growth inhibition mediated by effector cells from *C. neoformans*-treated mice was previously attributed to a Thy-1⁺ Ia⁺ lymphocyte population (7). At the time of that study, reagents specific for T cells such as monoclonal antibody to CD3, a molecule associated with the TCR, were unavailable. In the present study, the effector cell populations were phenotypically characterized with the T-cell-specific reagent, an anti-CD3 monoclonal antibody. Comparable cell populations isolated on day 8 after treatment of mice with SPSS, *C. neoformans* cells, or nothing were similar with respect to the percentages of T cells (CD3⁺ cells), B cells (μ^+ cells), NK cells (asialo-GM-1⁺ cells), and macrophages (cells staining positive for esterase). Representative results from cell phenotyping are shown in Table 1. T lymphocytes comprised about 22 to 23% of the SPC. By passage of the SPC over nylon wool, we achieved a significant enrichment of splenic T cells in the NWN-SPC fraction

TABLE 1. Analysis of effector cell populations^a

Cell population	% of cells with surface expression of ^b :					% Monocyte/macrophages ^c
	CD3	$\alpha\beta$ TCR	$\gamma\delta$ TCR	Asialo-GM-1	μ	
SPC	22–23	20–23	<1	2–5	ND ^d	ND
NWN-SPC	89–93	88–92	1–3	3–6	<1	<1
LN	78–83	72–75	1–3	<1	<1	ND
NWN-LN	98–99	98–99	1–3	1–3	<1	<1

^a Data are for effector cells isolated from untreated mice but were not significantly different from data acquired with cells from mice treated 8 days previous with SPSS or *C. neoformans*.

^b As determined by immunostaining and flow cytometry analysis.

^c As determined by nonspecific esterase staining.

^d ND, not done.

(89 to 93% T cells). In contrast to the SPC, LN cell pools contained 78 to 83% T cells, and after passage over nylon wool, the NWN-LN were 98 to 99% T lymphocytes. These data demonstrate that the NWN-LN populations were almost pure T lymphocytes (contamination of 1 to 2% with a combination of μ^+ or B cells, NK cells, and macrophages) whereas the NWN-SPC had 1% B-cell and macrophage contamination and 3 to 6% asialo-GM-1⁺ cell contamination (NK cells). A portion of the asialo-GM-1⁺ cells could have been cytotoxic T lymphocytes (CTL), since CTL are also asialo-GM-1⁺ (20, 37). Considering the phenotyping data in conjunction with the growth inhibition data, the 40% inhibition of cryptococcal growth observed with the NWN-LN populations which were 98 to 99% CD3⁺ cells strongly indicates that the observed cryptococcal growth inhibition was mediated by T lymphocytes. This idea is supported also by the fact that as the cell populations were enriched for T lymphocytes, the anticryptococcal activity of the effector cell populations concomitantly increased (Fig. 1 and Table 1).

Binding of effector cells to viable *C. neoformans*. Murine NK cells and human NK and T cells have been shown to be capable of binding to cryptococcal cells (33, 34, 36). Effector cell binding to a target cell is the first step in cytotoxic lymphocyte-mediated killing of a target cell (2, 23, 41). To demonstrate that the cell-cell contact was a characteristic of murine T-cell interactions with *C. neoformans*, we next determined the ability of murine SPC and LN from immunized and saline-treated mice to form conjugates with *C. neoformans* after a 2-h incubation. With SPC as an exception, effector cells from mice immunized with *C. neoformans* formed a higher percentage of conjugates with *C. neoformans* cells than did the respective cell populations from SPSS-treated mice (Fig. 2).

The time required for effector cell-*C. neoformans* conjugates to form was brief. Conjugates were observed at the earliest time point that assessments were made, and peak numbers of conjugates were detected after approximately a 15-min incubation of cryptococci with lymphocytes (Fig. 3). The time course for conjugate formation was the same for lymphocytes from immunized mice as well as from SPSS-treated mice; however, at all time points, a significantly greater percentage of conjugates formed with NWN-SPC from immunized mice than with NWN-SPC from control mice (Fig. 3).

Cryptococcal growth inhibition is due to T lymphocytes. Since NWN-SPC populations contained 3 to 6% asialo-GM-1⁺ cells, we depleted mice of asialo-GM-1⁺ cells by injecting mice 1 day before treatment with anti-asialo-GM-1; then on day 8 after treatment, the NWN-SPC were obtained. NWN-SPC populations from mice treated intravenously with normal rabbit serum contained 3.2 to 4% asialo-GM-1⁺ cells, whereas

FIG. 2. Mean percentages of lymphocytes of the designated cell populations that form conjugates with *C. neoformans* after 2 h of incubation. Error bars represent the standard errors of the means. *P* values from unpaired Student *t* tests for comparison of significance between the activities of effector cell populations isolated from SPSS- and from *C. neoformans*-treated mice are in parentheses. NS, not significant. Data are representative of three experiments.

NWN-SPC from mice treated with anti-asialo-GM-1 antibody had 0.5 to 1.1% asialo-GM-1⁺ cells (Table 2). The treatment of mice with anti-asialo-GM-1 antibody also ablated the NK activity of the NWN-SPC populations, as indicated by the lack of ⁵¹Cr release from YAC-1 target cells (Table 2). Depletion of NK cells and NK cell activity from the cell populations did not alter the anticytotoxic activity of the NWN-SPC populations (Table 2), indicating that NK cells were not responsible for the inhibition of the cryptococcal cell growth.

Experiments similar to the *in vivo* depletion of asialo-GM-1⁺ cells with anti-CD3, anti- α/β TCR, or a combination of anti-CD4 and anti-CD8 antibodies in place of anti-asialo-GM-1 antibody were not feasible, because the procedures result in so few cells in the purified lymphocyte fractions that the *in vitro* binding and growth inhibition assays cannot be performed. To draw valid conclusions from the conjugation and growth inhibition assays, it is necessary to use the same E/T ratio and the same numbers of effector and target cells in the assay wells for each group of cells being tested. Furthermore, if one depletes mice of T cells or α/β TCR⁺ cells and obtains the NWN-SPC fraction, the remaining cells will be predominantly NK cells which have anticytotoxic activity. The NK cell-enriched populations from the T cell- or α/β TCR⁺ cell-depleted mice would display anticytotoxic activity in the *in vitro* assays. Consequently, one would not be able to interpret the data from such experiments to make a statement regarding T-lymphocyte activity against *C. neoformans*.

Growth inhibition by effector cells requires cell-cell contact. Although results of experiments with murine NK cells and *C. neoformans* have established that growth inhibition of *C. neoformans* does not occur if NK cells-*C. neoformans* conjugate formation is blocked (12, 32), definitive studies have not been done to determine if direct cell-cell interactions between murine T lymphocytes and *C. neoformans* are necessary for cryptococcal growth inhibition to occur. Because *C. neoformans* grows as well in filtered spent TCM from wells of a growth inhibition assay in which inhibition of *C. neoformans* growth had occurred as it does in fresh TCM, it seems unlikely that a stable, soluble factor(s) was mediating the anticytotoxic activity. However, a labile, soluble factor(s) could be responsible for the cryptococcal growth inhibition. This was ruled out, however, by the fact that no growth inhibition of *C. neoformans* was observed when *C. neoformans* cells and effector cells were separated by a membrane in Transwell plates (Table 3). To determine if the absence of cryptococcal growth inhibition

FIG. 3. Mean percentages of NWN-SPC populations from SPSS- or *C. neoformans*-treated mice that form conjugates with *C. neoformans* after various incubation lengths. Error bars represent the standard errors of the means. *P* values from unpaired Student *t* tests for comparison of significance between the percentages of effector cells isolated from SPSS- and from *C. neoformans*-treated mice that bound cryptococci at each time point were <0.001. Data are representative of three experiments.

observed in the Transwell plates was the result of death of the effector cells, the NWN-SPC were removed from the upper Transwell compartment after the 24-h incubation, and the viability of the effector cells was determined by trypan blue dye exclusion and found to be consistently between 91 and 96%. Moreover, the effector cells did not lose their ability to inhibit the growth of *C. neoformans* during the 24-h incubation in the Transwell plate assay, because when effector cells were removed from the Transwells and put into wells of a 96-well plate in direct contact with cryptococcal cells, the effector cells were able to inhibit cryptococcal growth to a similar level as effector cells from the same cell pool that had not been previously incubated in the Transwells (Table 4). When the effector cells were not separated from the *C. neoformans* cells by the porous membrane in the Transwell plates, effector cells from SPSS- and *C. neoformans*-treated mice inhibited the growth of cryptococcal cells by 34.2 and 48.7%, respectively. Together these data document that inhibition of cryptococcal growth by murine T cells is dependent on direct contact between the effector cells and the cryptococcal cells.

Lymphocytes are cidal for *C. neoformans*. To determine whether the cryptococcal growth inhibition mediated by T lymphocytes was the result of killing of cryptococcal cells or the result of prevention of proliferation of cryptococcal cells, the viability of cryptococcal cells was assessed with a vital stain on the basis of percentage of viable *C. neoformans* in the total *C.*

TABLE 2. Effects of *in vivo* depletion of NK cells on the direct anticytotoxic activity of NWN-SPC from control and immune mice^a

In vivo treatment of mice		% of asialo-GM-1 ⁺ cells	% ⁵¹ Cr released from YAC-1 target cells	% Growth inhibition of viable <i>C. neoformans</i> cells
Day -1 (i.v.)	Day 0 (s.c.)			
NRS	SPSS	4.0	2.8 ± 1.4	37.4 ± 1.3
Anti-asialo-GM-1	SPSS	1.1	-2.9 ± 0.6	40.0 ± 1.7
NRS	HK-Cn	3.2	5.0 ± 2.7	61.0 ± 2.3
Anti-asialo-GM-1	HK-Cn	0.5	1.7 ± 1.7	56.0 ± 4.5

^a NWN-SPC were collected 8 days after treatment of mice with SPSS or heat-killed *C. neoformans* cells (HK-Cn). i.v., intravenously; s.c., subcutaneously; NRS, normal rabbit serum.

TABLE 3. Growth inhibition of *C. neoformans* analyzed by using Transwell inserts^a

Group	Treatment of donor mice	Cell population		% Growth inhibition	P value compared with:	
		Upper well	Lower well		Group 1	Group 3
1	SPSS	NWN-SPC	Cn	-1.5 ± 2.2		NS
2	SPSS	None	NWN-SPC + Cn	34.2 ± 2.5	<0.001	<0.001
3	HK-Cn	NWN-SPC	Cn	-8.3 ± 2.2	NS	
4	HK-Cn	None	NWN-SPC + Cn	48.7 ± 1.7	<0.001	<0.001

^a Cn, viable *C. neoformans*; NS, not significant; HK-Cn, donor mice treated with heat-killed *C. neoformans* cells.

neoformans population and on the basis of the viability of individual *C. neoformans* cells in conjugate formation with a lymphocyte. The percentage of viable cryptococci in the total cryptococcal cell population in each well was microscopically determined by counting in a minimum of 200 *C. neoformans* cells the number of cryptococcal cells containing the purple MTT-formazan crystals (live cryptococci). In control wells which contained cryptococcal cells alone, the viability of the organism by this method was routinely 90 to 95% after a 24-h incubation. In contrast, under similar conditions when *C. neoformans* cells were incubated with lymphocyte populations, killing of the cryptococci was observed (Fig. 4). There was a significant increase in the percentage of dead cryptococci in wells containing NWN-SPC from *C. neoformans*-immunized mice compared with wells containing NWN-SPC from SPSS-treated mice (Fig. 4A). The percentage of cryptococci killed by immune NWN-LN which were 98% CD3⁺ cells was slightly greater than the percentage of *C. neoformans* killed by immune NWN-SPC which were 89 to 93% CD3⁺ cells (Fig. 4A).

Because cell-cell contact is required for growth inhibition of *C. neoformans* by T lymphocytes, the percentage of killed cryptococci bound by effector cells was determined (Fig. 4B). The population of cryptococcal cells in direct contact with lymphocytes from immunized mice contained a higher percentage of dead cells (cryptococci free of purple formazan crystals) than was observed in populations of *C. neoformans* cells bound to effector cells from control mice (Fig. 4B). The profiles of anticytotoxic activity of the four different effector cell populations were similar whether assessment was made with *C. neoformans* viability determinations with MTT or with the cryptococcal growth inhibition assessment by CFU counts (compare data in Fig. 4A or B with data in Fig. 4C).

DISCUSSION

With a microassay for the measurement of cryptococcal growth inhibition by effector cell populations, we confirmed the results of Fung and Murphy (7) that murine T lymphocytes from *C. neoformans*-immunized mice inhibit *C. neoformans* growth more effectively than do similar effector cell populations from saline-treated mice. Slightly different parameters were used in the investigation of Fung and Murphy (7) than in

the study presented here. For instance, Fung and Murphy (7) used a tube assay system and E/T ratios ranging from 100:1 to 500:1 to show that murine Thy1⁺ Ia⁺ lymphocytes were the anticytotoxic effector cells in the spleens of immunized mice. In the present investigation, we used a microassay with an E/T ratio of 2:1 and demonstrated that CD3⁺ αβ TCR⁺ lymphocytes were the cells mediating the anticytotoxic activity. Even though there were different parameters in the two studies, the results are in agreement. From both studies, it can be concluded that murine T lymphocytes are mediators of anticytotoxic activity.

The observation that normal humans have peripheral blood T lymphocytes which bind and inhibit the growth of *C. neoformans* (24, 33) caused us to question whether those individuals had been previously exposed to *C. neoformans*. The data presented here clearly demonstrate that saline-treated mice have T lymphocytes which can limit the growth of cryptococci. This was evident because an increase in anticytotoxic activity was concomitant with the enrichment of T lymphocytes from lymph nodes of mice injected with SPSS. We also observed that approximately 13 to 20% of the cells in the populations of 98 to 99% αβ T cells from LN of control mice formed conjugates with *C. neoformans* cells. This is probably a conservative estimate of the percentage of T cells which bind *C. neoformans* cells, because when the E/T ratio is changed to 1:1, the percentage of effector cells binding cryptococcal targets is significantly increased. Furthermore, the highly purified T-cell populations from SPSS injected mice displayed anticytotoxic activity, albeit at a lower level than similar cell populations from immune mice. The concept that naive mice have T lymphocytes with direct anticytotoxic activity is supported also by two additional observations. First, removal of NK cells by treating mice with anti-asialo-GM-1 antibody did not abrogate the anticytotoxic activity of the NWN-SPC obtained from the SPSS- and anti-asialo-GM-1-treated mice (Table 2). Second, NWN-SPC from SPSS-treated SCID mice, which lack B and T cells, do not inhibit the growth of *C. neoformans* (0.0% ± 3.5% growth inhibition), whereas NWN-SPC from BALB/c control mice show 21.7% ± 2.4% *C. neoformans* growth inhibition (35a). Moreover, immunization of the SCID mice with heat-killed cryptococci did not augment the anticytotoxic activity of the splenic NWN cells (0.4% ± 2.3% *C. neoformans*

TABLE 4. Growth inhibition of *C. neoformans* by NWN-SPC in 96-well plates^a

Group	Treatment of donor mice	Previous incubation in Transwell plate	% Growth inhibition	P value compared with:	
				Group 1	Group 3
1	SPSS	—	23.3 ± 1.5		0.005
2	SPSS	+	18.8 ± 1.8	NS	0.001
3	HK-Cn	—	39.5 ± 2.2	0.005	
4	HK-Cn	+	36.3 ± 2.8	0.015	NS

^a NS, not significant; HK-Cn, donor mice treated with heat-killed *C. neoformans* cells.

FIG. 4. Mean percentages of *C. neoformans* killed and *C. neoformans* growth inhibition after incubation with the designated effector cell populations. (A) Total percentage of cryptococci killed (bound and unbound by effector cells) after a 24-h incubation; (B) percentage of killed cryptococci that were bound by effector cells after a 24-h incubation; (C) cryptococcal growth inhibition after a 24-h incubation. Error bars represent the standard errors of the means. *P* values from unpaired Student *t* tests for comparison of significance between the activities of effector cell populations isolated from SPSS- and from *C. neoformans*-treated mice are in parentheses. Data are representative of three experiments.

growth inhibition), but a similar immunization did increase the anticytotoxic activity of NWN-SPC from BALB/c mice ($39.4\% \pm 2.1\%$ *C. neoformans* growth inhibition) (35a). Considered together, our findings indicate that T lymphocytes from nonimmune mice have direct anticytotoxic activity.

Although the NWN-SPC populations used in our experiments had 3 to 6% NK cell contamination, one cannot attribute the observed anticytotoxic activity to NK cells because removal of NK cells by *in vivo* depletion with anti-asialo-GM-1 did not alter the anticytotoxic activity even though the treatment ablated NK cell activity. The fact that anticytotoxic activity increased as the control SPC populations were enriched for T lymphocytes further supports the notion that nonsensitized mice have T lymphocytes which can directly interact with cryptococcal cells and limit cryptococcal growth. Our data show that immunization with *C. neoformans* cells augments the T-cell-mediated anticytotoxic activity in both lymph nodes and spleens. From our findings in the mouse model, one might predict that some if not all of the anticytotoxic activity reported by us and others (24, 25, 33) for freshly isolated human T lymphocytes from normal blood donors is mediated by nonsensitized T cells. However, since *C. neoformans* is ubiquitous in nature, it is possible that humans

with exceptionally high levels of T-cell-mediated fungistatic activity against *C. neoformans* have been previously sensitized to the organism.

Both major histocompatibility complex (MHC)-restricted and -nonrestricted cytotoxic T cells must bind to their target cells prior to inducing their lethal effect on the target cell (2, 23, 40, 41), so we were interested in whether murine T lymphocytes that mediate anticytotoxic activity conform to this general characteristic of cytotoxic lymphocytes. We performed two different studies to address this issue. First, we showed that about 12 to 15% of the T lymphocytes from SPSS-treated mice and 32 to 38% of the T lymphocytes (98 to 99% pure T-cell populations) from *C. neoformans*-immunized mice bind to cryptococcal cells. Second, we demonstrated that if direct contact between the cryptococcal cells and the lymphocytes was prevented, as in the Transwell plates, anticytotoxic activity did not occur. The lack of anticytotoxic activity in the Transwell plates was not due to trivial reasons. For instance, Transwell plates did not prevent the demonstration of anticytotoxic activity because when effector and target cells were incubated together in the lower chambers of the Transwell plates, inhibition of *C. neoformans* growth occurred. Furthermore, the viability of lymphocytes that had been incubated for 24 h in the upper chambers of the Transwell plates with *C. neoformans* in the lower chambers was not diminished (91 to 96% viable), and those lymphocytes were able to inhibit the growth of *C. neoformans* when cultured directly with fresh viable *C. neoformans* cells for another 24 h in 96-well plates. These results show that the effector cells were viable and able to function in the anticytotoxic assay after having been subjected to the Transwell plates. After ruling out trivial explanations for the lack of anticytotoxic activity when effector cells were separated from *C. neoformans*, in the Transwell plate studies, we concluded that murine T lymphocytes must bind to *C. neoformans* cells to mediate direct inhibition of cryptococcal cell growth.

The ability of *C. neoformans* to proliferate after incubation with or without lymphocytes was the standard assay that we used to detect anticytotoxic activity. Consequently, we have presented the results from such assays as inhibition of cryptococcal growth. The assessment of CFU does not allow us to distinguish between fungicidal effects on cryptococcal cells (dead cells) and fungistatic effects (cryptococcal cells are viable but unable to proliferate). To distinguish between live cryptococcal cells with active metabolic functions (mitochondrial dehydrogenases) and dead cells which lack active mitochondrial dehydrogenases, we treated the cell mixtures with MTT, a vital stain that is converted to an insoluble purple compound in viable cells. Our data show that both at the single-conjugate level and in the total suspensions of *C. neoformans* and effector cells, the *C. neoformans* cells are killed as a result of the T-lymphocyte interactions. The results from vital staining experiments are similar to those from CFU determination studies, indicating that T-cell-mediated growth inhibition is due primarily to cryptococcal cell death.

Our data in conjunction with the findings of Fung and Murphy (7) suggest that T lymphocytes may play a role in first-line defense against *C. neoformans* by direct binding and killing of the cryptococcal cells. This direct T-cell-mediated mechanism as a first-line defense can clearly be upregulated by immunization of mice, so one might speculate further that if individuals have been previously sensitized or immunized with *C. neoformans*, then their T lymphocytes would have increased capacity to eliminate *C. neoformans* through direct interactions. These speculations do not preclude the possibility that T lymphocytes in the *C. neoformans*-immune animals also may

function indirectly via lymphokine production to activate other cellular components and eliminate the organism. In immunologically normal individuals who develop CMI responses to *C. neoformans*, one would expect both direct and indirect effects of T lymphocytes on cryptococcal cells to contribute to host defense.

CTL are frequently described as important protective components of an immune response in viral infections (2, 23, 41). Virus-specific CTL via their TCR recognize and bind viral antigen associated with MHC class I and some times MHC class II antigens on the surface of the virus-infected host cells (2, 23, 41). Direct T-lymphocyte interactions with extracellular microorganisms have been reported, but much less frequently than have CTL directed at virus-infected cells. Furthermore, the direct antimicrobial interactions may not be mediated through a TCR-antigen-MHC interaction which is typical of an interaction with virus-infected cells. As previously mentioned, Fung and Murphy (7) in 1982 reported that immunization with intact *C. neoformans* cells resulted in the induction of splenic and lymph node T cells that were capable of inhibiting cryptococcal growth in vitro and of transferring protection to naive mice against a *C. neoformans* challenge. T-lymphocyte interactions with extracellular microbial pathogens are not unique to *C. neoformans*. Direct interactions of T lymphocytes from mice and humans have been observed with several parasitic organisms. For instance, Salata et al. (38) showed that T lymphocytes isolated from patients with amebic liver abscesses when stimulated in vitro with amebic proteins were activated to kill virulent *Entamoeba histolytica* amebae, whereas lymphocytes from normal donors similarly treated did not kill the amebae. Khan et al. (18) identified in splenic cell populations from *Trypanosoma gondii*-immunized mice *T. gondii*-specific CD4⁺ CD8⁺ T lymphocytes that were directly parasitocidal to extracellular *T. gondii*. More recently, Khan and coworkers (19) have cloned T cells from *T. gondii*-seropositive individuals and demonstrated that one of their human $\alpha\beta$ TCR⁺ T-cell clones exhibits direct cytotoxicity in a non-MHC-restricted manner on extracellular *T. gondii*. Human $\gamma\delta$ TCR⁺ T lymphocytes have been reported to inhibit replication of extracellular merozoites of *Plasmodium falciparum* (6). In our model, we can rule out the possibility that the T lymphocytes interacting with cryptococci are all $\gamma\delta$ TCR⁺ cells because the percentage of $\gamma\delta$ TCR⁺ cells in the mouse T-lymphocyte populations that we studied in direct interactions with *C. neoformans* was very low (1 to 3%); the bulk of our T lymphocytes was $\alpha\beta$ TCR⁺. Since the conventional MHC-restricted interaction of a TCR with antigen requires binding of the TCR to antigen and to the self-MHC molecule holding the antigen, the direct interaction of T cells with cryptococcal cells would be considered non-MHC restricted because cryptococcal cells do not have murine MHC or surface molecules which will bind to fluorescently labeled anti-I-A^k or anti-I-A^k antibodies (30a). Furthermore, one would not expect 12 to 15% of murine T cells to have TCR with antigen binding grooves specific for cryptococcal antigens, yet 12 to 15% of the T lymphocytes in the cell populations from saline-treated mice bind cryptococcal cells. Thus, it seems reasonable to predict that at least some of the T lymphocytes binding to cryptococci do so through lymphocyte surface receptors other than the *Cryptococcus*-specific TCR, or if binding is through the TCR, it does not occur in a conventional manner. Consequently, it appears that the binding of *C. neoformans* to T cells is non-MHC restricted and not solely dependent on interactions through the $\alpha\beta$ TCR.

Our data add to the growing amount of evidence that lymphocytes can directly interact with some extracellular microbial pathogens and limit the growth or kill the microbe. Natural

killer cells from both mice and humans have been shown to bind to *C. neoformans* and to prevent growth of the organism or to kill the cryptococcal cells (14, 25, 33, 34, 36). Killing of the cryptococcal cells by NK cells is mediated at least in part by perforin contained in the granules of the NK cells (13). Cytotoxic T-cell subsets are similar to NK cells in that they have granules which contain perforin and serine esterases (41). The data presented here demonstrate that murine T lymphocytes can bind and kill *C. neoformans* target cells, so in light of the similarities in killing mechanisms between NK cells and cytotoxic T cells, it is tempting to speculate that the T cells cytotoxic to *C. neoformans* kill the organism in a manner similar to the way murine NK cells kill cryptococci, i.e., by focused exocytosis of perforin-laden granules toward the bound cryptococcal cell (11).

In summary, our data indicate that normal as well as immune animals have $\alpha\beta$ TCR⁺ T cells that can directly kill *C. neoformans* cells. Since anticryptococcal activity mediated by cytotoxic T cells is augmented by immunization with heat-killed organisms, it may be possible to develop a vaccine protocol that will provide two types of host protection against *C. neoformans*, one via direct interactions of the T lymphocytes with cryptococcal cells and the other via indirect activities of T lymphocytes which might be through the production of lymphokines that activate natural effector cells.

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