Direct Activity of Human T Lymphocytes and Natural Killer Cells against *Cryptococcus neoformans*

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Lymphocytes constitute a critical component of host defenses against cryptococcosis. Previously, we demonstrated that human lymphocytes cultured with interleukin-2 formed conjugates with, and directly inhibited the growth of, *Cryptococcus neoformans*. Here, we explore the anticryptococcal activity of freshly isolated, highly purified populations of human peripheral blood lymphocytes. Lymphocytes were incubated with encapsulated *C. neoformans* for 24 h, after which the lymphocytes were lysed, dilutions and spread plates were made, and CFU were counted. Fungistasis was determined by comparing growth in wells with and without lymphocytes. Nylon wool-nonadherent peripheral blood mononuclear cells (NWNA PBMC) were highly fungistatic, even if either T cells or natural killer (NK) cells were depleted by panning. A mixed population of T cells and NK cells, obtained by rosetting NWNA PBMC with sheep erythrocytes, completely inhibited cryptococcal growth, whereas the nonrosetting cells had little fungistatic activity. CD4$^+$, CD8$^+$, and CD16/56$^+$ lymphocytes, isolated by positive immunoselection, had potent growth-inhibitory activity. In contrast, purified B cells had no activity. Fungistasis was seen even in the absence of opsonins. Antifungal activity was markedly diminished when surface receptors on NWNA PBMC were cleaved by treatment with trypsin or bromelain. Supernatants from stimulated lymphocytes or concentrated lymphocyte sonicates were not active. Lymphocyte-mediated fungistasis was seen with two different strains of *C. neoformans*. CD4$^+$, CD8$^+$, and CD16/56$^+$ lymphocytes formed conjugates with *C. neoformans*, as observed under Nomarski differential interference contrast microscopy and videomicroscopy. These data demonstrate that freshly isolated peripheral blood T cells and NK cells have the capacity to bind and directly inhibit the growth of *C. neoformans*.

The yeast *Cryptococcus neoformans* has a marked propensity to cause infections in patients with impaired T-cell function, such as those with AIDS, lymphoreticular malignancy, and idiopathic CD4$^+$ lymphocytopenia and recipients of corticosteroid therapy (3, 5, 19, 34). In particular, cryptococcosis has emerged as one of the most frequent life-threatening infections in patients with AIDS, afflicting at least 5 to 10% of AIDS patients in the United States and an even higher percentage in parts of the developing world (3, 19, 34). The strong association of human cryptococcosis with disorders of T lymphocytes (especially CD4$^+$ T cells) points to a critical role for this cell type in cryptococcal host defenses.

In murine models of cryptococcosis, pulmonary inoculation of *C. neoformans* results in an influx of both CD4$^+$ and CD8$^+$ T cells. Mice depleted of either CD4$^+$ or CD8$^+$ cells have been noted to have impaired clearance and increased susceptibility to systemic dissemination when challenged intratracheally with *C. neoformans* (11, 12, 14, 29). How the T lymphocyte contributes to the immune response against *C. neoformans* remains incompletely defined and is probably multifactorial. Cytokines (e.g., gamma interferon and interleukin-4 [IL-4]) secreted by activated T lymphocytes may capacitate macrophages and possibly other cell types for increased fungicidal capacity and/or confinement of the yeast within multinucleated giant cells (6, 11, 20). Recently,

we demonstrated another potential mechanism by which T cells may promote host defenses against cryptococcosis: highly purified IL-2-activated human lymphocytes expressing T-cell or natural killer (NK) cell surface markers formed conjugates with and directly inhibited the growth of *C. neoformans* in the absence of MHC restriction (22). In the present study, we examine the anticryptococcal activity of freshly isolated human peripheral blood T cells and NK cells.

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MATERIALS AND METHODS

Materials. Chemicals were obtained from Sigma Chemical Co. (St. Louis, Mo.) except where otherwise noted. The cell culture medium was RPMI 1640 (GIBCO, Grand Island, N.Y.) supplemented with L-glutamine, penicillin, streptomycin, and 10% human AB male serum. Flat-bottomed 96-well "half-area" polystyrene tissue culture plates (model 3696; Costar, Cambridge, Mass.) were used for assays involving cell wells. Pooled human serum (PHS) was obtained by combining serum from 12 healthy donors under conditions that preserved complement activity. The PHS contained low levels of anticapsular (antiglucuronoxylomannan) antibody of the immunoglobulin G (IgG), IgM, and IgA classes, as measured by a sensitive enzyme-linked immunosorbent assay in the laboratory of Thomas Kozel (Reno, Nev.). Recombinant human IL-2 was a gift of Cetus Corporation (Emeryville, Calif.).

C. neoformans. Two well-described encapsulated crypto-

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coccal strains were used: serotype D strain B3501 (18, 22, 25), obtained from Eric Jacobson, Medical College of Virginia, Richmond, Va.; and serotype A strain 145 (28), obtained from Thomas Mitchell, Duke University Medical Center, Durham, N.C. *C. neoformans* were harvested from 4-day-old cultures grown on asparagine minimal agar medium at 30°C, as in previous studies (18, 22, 25). Under these conditions, over 90% of the *C. neoformans* was present as single cells, and clumping of organisms was not observed (23).

Isolation of PBMC. Human peripheral blood was obtained by venipuncture from normal volunteers. More than 10 donors were used. For each set of experiments, blood from the same donor was not used more than once. Blood was anticoagulated with heparin, and the peripheral blood mononuclear cells (PBMC) were purified by centrifugation on a Ficoll-Hypaque density gradient. PBMC were washed three times in phosphate-buffered saline (PBS) containing 0.1% bovine serum albumin, counted, and suspended in medium at the desired concentration (18).

Nylon wool columns. Nylon wool-nonadherent (NWNA) PBMC were prepared as in previous studies by allowing cells to adhere to plastic tissue culture petri dishes for 1 h and then passing nonadherent cells over a nylon wool column (15, 22).

SE rosettes. PBMC were separated into two populations by their ability to rosette with neuraminidase-treated sheep erythrocytes (SE; Cappel, West Chester, Pa.) as in previous studies (22, 33). T cells and a subpopulation of NK cells express CD2, the SE receptor, and thus form rosettes with SE (38). Ficoll-Hypaque density gradient centrifugation was used to separate rosetting cells from nonrosetting cells (hereafter referred to as the rosette and interface fractions, respectively). Cells in the rosette fraction were rid of SE by lysis with hypotonic saline.

Panning. Negative and positive selection of cells by panning specific PBMC subpopulations for cell surface markers was done exactly as described before (22). Briefly, monoclonal antibody (MAb)-coated cells were gently centrifuged on petri dishes coated with affinity-purified goat anti-mouse IgG (whole molecule) (Cappel). For negative selection experiments, after a 70-min incubation at 4°C, the nonadherent cells were collected and panned a second time. Controls consisted of sham-panned cells, which underwent the entire panning procedure except that they were not incubated with MAb. For positive selection of lymphocyte subpopulations, cells which remained adherent after five washes were cultured directly in the petri dish. After 18 h, the positively selected cells (which at this point were nonadherent or loosely adherent) were collected after gentle agitation and washed to remove free antibody. Controls consisted of cells which were not treated with MAb but cultured for 18 h in petri dishes.

LME treatment and isolation of B cells. The protocol of Splawski and Lipsky was used to isolate B cells (37). Briefly, 3×10^6 PBMC/ml were incubated in a freshly prepared solution of PBS containing 5 mM leucine methyl ester (LME) for 35 min at room temperature. Cells were then washed three times and filtered through fine nylon mesh to remove clumps and debris. B cells were separated from residual T cells by rosetting with neuraminidase-treated SE, as described above.

Supernatants and cell lysates. Supernatants were collected from wells containing 10^6 NWNA PBMC with or without 5×10^3 *C. neoformans* for 24 h in 150 μl of medium supplemented with 10% PHS. Lysates were prepared by sonicating

NWNA PBMC (5×10^7/ml in RPMI 1640) at 0°C under conditions shown to completely disrupt the cells. Lysates and supernatants were stored at −70°C until use.

Protease treatment. NWNA PBMC were incubated with trypsin and bromelain under conditions shown previously to cleave cell surface receptors (8, 25). Cells (5×10^6/ml) were incubated with 100 μg of trypsin per ml for 30 min at 37°C and then treated with 100 μg of soybean trypsin inhibitor per ml. The cells were then washed three times in RPMI 1640, recounted, and suspended at the desired concentration. Treatment with bromelain (1 mg/ml) was performed exactly as for trypsin except that the incubation was extended to 1 h and trypsin inhibitor was not added.

Cell culture. For experiments examining the effects of IL-2, effector cells (10^6/ml) were cultured in 75-cm^2 tissue culture flasks containing medium for up to 7 days. During this incubation period, cells were either stimulated with 1,000 U of IL-2 per ml or left unstimulated. Cells were then harvested, washed, counted, and resuspended at 10^7/ml in fresh medium with and without IL-2.

Antifungal activity of cultured PBMC. Antifungal activity was determined as described previously (18, 22–24). Cell wells contained 1×10^6 or 2×10^5 effector cells and 5×10^3 CFU of *C. neoformans* in a final volume of 150 μl of medium supplemented with 10% (final concentration) PHS. For some cell wells, as indicated, serum was either omitted from the system or heat inactivated (56°C for 30 min). Wells were incubated for 24 h at 37°C in humidified air supplemented with 5% CO_2, after which the number of CFU of *C. neoformans* per well was determined by lysing the effector cells with 0.1% Triton X-100 and then making dilutions and spread plates on Sabouraud dextrose agar. Preliminary experiments established that this procedure completely lysed effector cells, eliminated clumping, and did not affect fungal viability.

For each experiment, two sets of cell wells containing *C. neoformans*, medium, and PHS but no effector cells were included. The first set was detergent treated, diluted, and plated immediately. The CFU counted were used to calculate the inoculum of live organisms added per well. The second set was incubated at 37°C for 24 h before being processed and plated. The CFU counted were used to calculate fungal replication in medium not containing effector cells.

Results are expressed as percent growth according to the following formula: [(experimental CFU/inoculated CFU) − 1] × 100. Thus, a value of zero indicates that the number of CFU at the start and conclusion of the incubation was the same and that no net fungal growth occurred. Values of 100, 200, 400, and 800% indicate that the fungi underwent an average of 1, 2, 3, and 4 replications, respectively. A negative value indicates that there was a decrease in CFU during the course of the incubation and therefore that fungal killing had taken place (18, 22–24).

Detection of cell surface antigens. Expression of specific cell surface markers was determined by flow cytometric analysis (FACScan; Becton Dickinson, Mountain View, Calif.) of fluorescein-stained cells (22, 24). The MAb used to detect the indicated cell surface antigens were as follows: T3 (CD3, T cells); T4 (CD4, T-cell subset), T1 (CD5, T cells and B-cell subset); T8 (CD8, T-cell subset and lymphokine-activated killer cell [LAK] subset); MY4 (CD14, monocytes and macrophages); anti-leu-11b (CD16, NK cells and LAK cells); B1 (CD20, B cells); and NKH-1 (CD56, NK cells and LAK cells). All MAb were obtained from Coulter Immunology (Hialeah, Fla.) except for anti-leu-11b, which was ob-
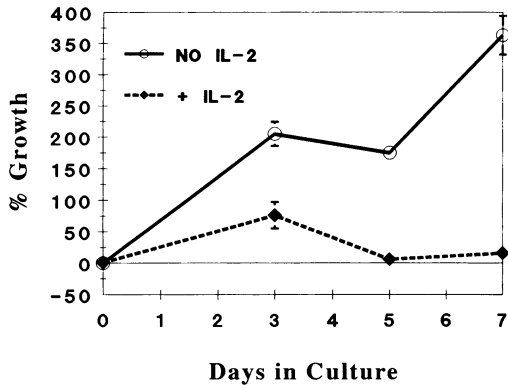
Days in Culture

FIG. 1. Fungistasis mediated by NWNA PBMC cultured for various periods with and without IL-2. NWNA PBMC (10^6 cells per well) were challenged for 24 h with *C. neoformans* B3501 either immediately after isolation (day 0) or after culture for 3, 5, or 7 days with or without 1,000 U of IL-2 per ml. Percent growth (see text for definition) was then assayed. Data represent the means ± SEM for three experiments performed in triplicate.

tained from Becton Dickinson. Included in each flow cytometry run were negative controls consisting of an isotypic control MAb and positive controls consisting of MAb W6/32 (a gift of Richard Maziarz, University of Oregon, Portland), which reacts with a public determinant on HLA-A, -B, and -C (24). Dead cells were gated out on the basis of positive staining with propidium iodide. For each sample, 10,000 events were recorded.

Nomarski DIC microscopy. Effector cells (5×10^4) and *C. neoformans* (2×10^4) were incubated for 1 h at 37°C in eight-chamber Lab-Tek slides (Miles Scientific, Naperville, Ill.) containing 200 μl of medium supplemented with 10% PHS. Cells were then fixed and observed at 1,000× magnification with a Nikon Diaphot-TMD inverted microscope (Nikon Corporation, Tokyo, Japan) equipped with Nomarski differential interference contrast (DIC) optics.

Videomicroscopy. Effector cells (5×10^4) and *C. neoformans* (2×10^4) were allowed to settle to the bottom of Lab-Tek slides containing a final volume of 200 μl of medium supplemented with 10% PHS and 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid, pH 7.4). Videomicroscopy was then performed in a 37°C humidified chamber with an inverted microscope connected to a videocamera (Panasonic CCTV model WV-1460) equipped with a zoom ocular. Events were videotaped with a Panasonic model AG-6010 professional videocassette recorder on time lapse mode, as described before (22).

Statistics. Means and standard error of the mean (SEM) for sample groups were compared by the two-sample, two-tailed Student *t* test. For experiments in which more than one comparison was made, the *P* value to determine significance was adjusted by using the Bonferroni correction.

RESULTS

Effect of IL-2. Previously, we demonstrated that NWNA PBMC cultured for 7 days with (but not without) IL-2 were fungistatic against cryptococcal strain B3501 (22). Here, we examined the antifungal activity of NWNA PBMC that were freshly isolated versus cells that were cultured for 3, 5, and 7 days with and without IL-2 (Fig. 1). In agreement with data recently reported by Murphy et al. (30), freshly isolated
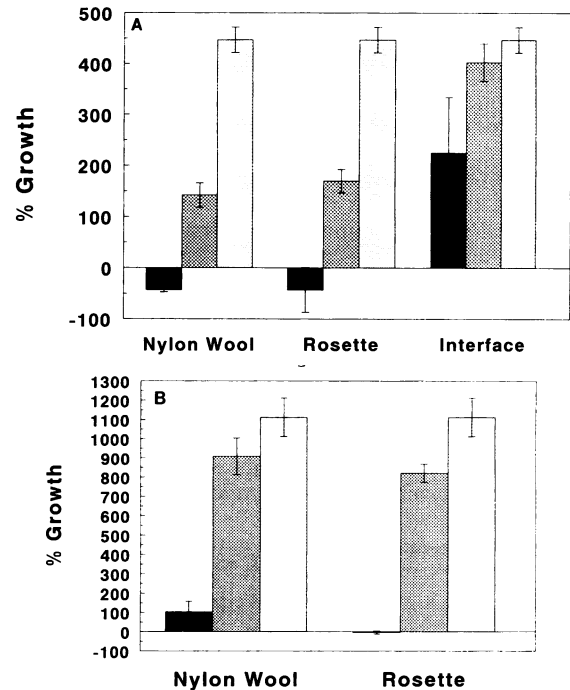

FIG. 2. Fungistasis mediated by NWNA PBMC fractionated by the ability to rosette SE. NWNA PBMC were either left unfractionated (nylon wool) or separated into fractions which rosetted (rosette) or did not rosette (interface) SE. *C. neoformans* was then added to wells containing 1×10^6 (solid bars), 2×10^5 (cross-hatched bars), or no (stippled bars) effector cells. After 24 h, fungal growth was assayed. Two fungal strains were tested, B3501 (A) and 145 (B). Insufficient cell yield precluded testing the interface fraction with strain 145. Data represent the means ± SEM for three experiments performed in duplicate or triplicate. For all groups, $P < 0.0001$ for no effector cells versus 10^6 cells except for the interface group, for which $P = 0.009$. For strain B3501, $P < 0.0001$ for no effector cells versus 2×10^5 cells in the nylon wool and rosette groups.

NWNA PBMC were intrinsically fungistatic. During culture without IL-2, this fungistatic capacity progressively waned. However, culture with IL-2 prevented the decline in fungistatic activity. Subsequent experiments focused on defining the component of the freshly isolated NWNA PBMC that was responsible for anticryptococcal activity.

Effect of rosetting. In the next set of experiments, NWNA PBMC were further fractionated by their ability to form rosettes with SE and then tested for activity against two strains of *C. neoformans*. As determined by flow cytometry, the rosetted cells averaged 79% T cells, 20% NK cells, 0% B cells, and 0% monocytes, while the cells in the interface (nonrosetting cells) averaged 0% T cells, 35% NK cells, 0% B cells, and 0% monocytes. (Approximately 65% of the cells in the interface did not stain for T, NK, B, or monocyte markers). The cells in the rosette fraction retained growth-inhibitory activity against both strains of *C. neoformans* tested, whereas cells in the interface had greatly diminished antifungal capacity (Fig. 2).

Negative selection by panning. The previous experiments, while strongly suggesting that T cells and/or NK cells had anticryptococcal activity, did not shed light on which population of cells was responsible. Therefore, in the next sets of experiments, the anticryptococcal activity of NWNA PBMC depleted of either NK cells or T cells by negative selection

TABLE 1. Expression of cell surface markers after negative selection by panning[a]

Cell fraction	Mean % of cells ± SEM expressing:					
	CD3	CD4	CD8	CD14	CD20	CD56
Sham-panned	81 ± 3	55 ± 2	29 ± 3	0	2 ± 0	7 ± 1
CD16/56 pan	91 ± 1	60 ± 1	29 ± 3	0	2 ± 1	1 ± 1
CD3/5 pan	0	ND[b]	ND	ND	ND	77 ± 5

[a] NWNA PBMC were either sham-panned or negatively selected by panning for the NK cell markers CD16 and CD56 or the T cell markers CD3 and CD5. The means ± SEM for seven (sham-panned), four (CD16/56 pan), and three (CD3/5 pan) separate experiments are given.

[b] ND, not determined.

TABLE 2. Expression of cell surface markers after positive selection by panning[a]

Cell fraction	Mean % of cells ± SEM Expressing:				
	CD3	CD4	CD8	CD14	CD56
No pan	94 ± 1	68 ± 2	25 ± 2	0	4 ± 2
CD4 pan	97 ± 1	93 ± 3	3 ± 1	0	0
CD8 pan	84 ± 5	5 ± 2	85 ± 7	0	9 ± 1
CD16/56 pan	35 ± 12	0	53 ± 29	ND[b]	42 ± 21

[a] NWNA PBMC were either cultured without panning or positively selected by panning for cells expressing CD4, CD8, or CD16 and CD56. The means ± SEM for three separate experiments are given.

[b] ND, not determined.

by panning was determined. NWNA PBMC that were sham-panned served as positive controls. As measured by flow cytometry, panning was highly effective (Table 1). Inhibition of fungal growth was seen regardless of whether the NWNA PBMC were depleted of T cells or NK cells (Fig. 3). However, growth-inhibitory activity against strain 145 was significantly greater when the NWNA PBMC were depleted of T cells than of NK cells. NWNA PBMC depleted of T cells by panning also had significantly greater lytic activity against the tumor target K562 (as measured by ^{51}Cr release [22]) than did NWNA PBMC that were either sham-panned or panned for NK cells (data not shown).

Positive selection by panning. In the next set of experiments, NWNA PBMC expressing CD4, CD8, or CD16 and/or 56 were positively selected by panning and then cultured overnight to allow detachment of cells and shedding of MAb. This technique yielded populations of CD4^{+} and CD8^{+} cells which were 93 and 85% pure, respectively (Table 2). Although only 42% of cells stained positive for CD56 after the CD16/56 pan, most of the cells which did not stain for CD56 had forward and side light scatter characteristics typical of NK cells (data not shown). Consistent with published observations (36), CD8 was found on 53% of the cells in the group panned for CD16/56. Positively selected cells were then tested for antifungal activity against strain 145. (Insufficient cell yields precluded testing strain B3501). Positively selected CD4^{+} lymphocytes, CD8^{+} lymphocytes, and CD16/56^{+} lymphocytes each had antifungal activity similar to that of NWNA PBMC cultured overnight without panning (Fig. 4).

Effect of LME and activity of B cells. The finding that both T lymphocytes and NK cells had anticryptococcal activity raised the possibility that all lymphocytes had anticryptococcal activity. To examine this possibility, PBMC were depleted of monocytes, NK cells, and cytotoxic T lymphocytes by treatment with LME (37, 39). The remaining cells were then incubated with neuraminidase-treated SE, and the cells forming rosettes (T cells) were separated from the nonrosetting cells by density gradient centrifugation. This
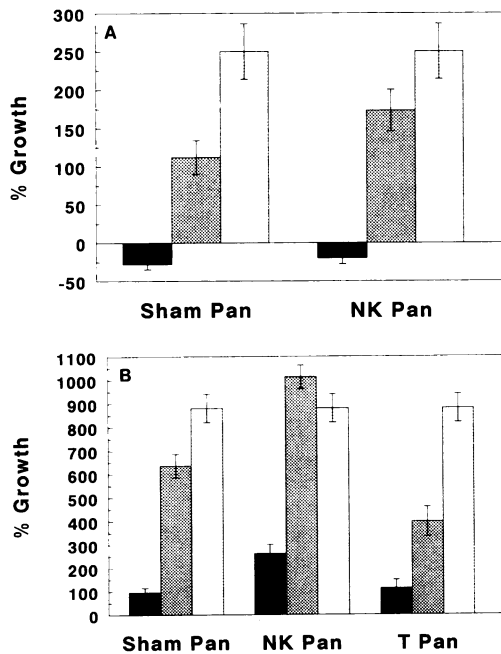

FIG. 3. Fungistasis mediated by NWNA PBMC negatively selected by panning. NWNA PBMC were either sham-panned or depleted of cells expressing the NK markers CD16 and CD56 (NK pan) or the T-cell markers CD3 and CD5 (T pan). The efficiency of panning is shown in Table 1. C. neoformans was then added to wells containing 1 × 10⁶ 10⁶ (solid bars), 2 × 10⁵ (cross-hatched bars), or no (stippled bars) effector cells. After 24 h, fungal growth was assayed. Two fungal strains were tested, B3501 (A) and 145 (B). Insufficient cell yield precluded testing the T pan fraction with strain B3501. Data represent means ± SEM for seven separate experiments, each performed in duplicate or triplicate. For all groups, P ≤ 0.0001 for no effector cells versus 10⁶ cells. For strain 145, P ≤ 0.002 for no cells versus 2 × 10⁵ cells in the sham pan and T pan groups.
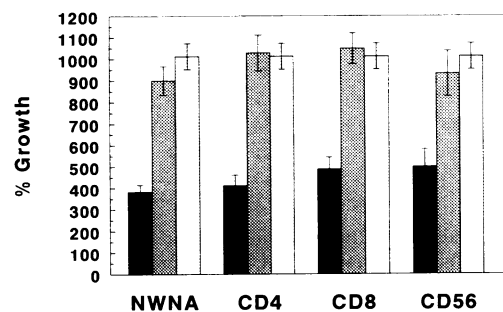

FIG. 4. Fungistasis mediated by lymphocyte subsets positively selected for by panning. NWNA PBMC were either cultured for 18 h without panning (NWNA) or positively selected for cells expressing CD4, CD8, or CD16/56. The efficiency of panning is shown in Table 2. C. neoformans strain 145 was then added to wells containing 1 × 10⁶ (solid bars), 2 × 10⁵ (cross-hatched bars), or no (stippled bars) effector cells. After 24 h, fungal growth was assayed. Data represent the means ± SEM for three experiments performed in triplicate. For all groups, P ≤ 0.0001 for no effector cells versus 10⁶ cells.

TABLE 3. Expression of cell surface markers after treatment
with LME and rosetting with SE

Cell fraction[a]	Mean % of cells ± SEM[b] expressing:					
	CD3	CD4	CD8	CD14	CD20	CD56
PBMC	58 ± 7	38 ± 5	22 ± 1	13 ± 5	10 ± 2	5 ± 4
LME-treated PBMC	85 ± 3	65 ± 8	17 ± 5	1 ± 1	12 ± 2	3 ± 3
Rosette	96 ± 2	80 ± 9	16 ± 6	0	1 ± 1	1 ± 1
Interface	0	0	0	0	83 ± 6	0

[a] See text for explanations.
[b] Mean ± SEM for three separate experiments.

procedure proved to be very efficient; 96% of the rosetting cells expressed the T-cell marker CD3, whereas 83% of the nonrosetting (interface) cells expressed the B-cell marker CD20 (Table 3). As expected, PBMC had excellent antifungal activity against strains B3501 and 145 (Fig. 5). Most of the antifungal activity was abolished after treatment with LME. When the LME-treated PBMC were further separated into T-cell and B-cell fractions by rosetting with SE, only modest antifungal activity was seen in the T-cell fraction and no significant activity was seen in the B-cell fraction.

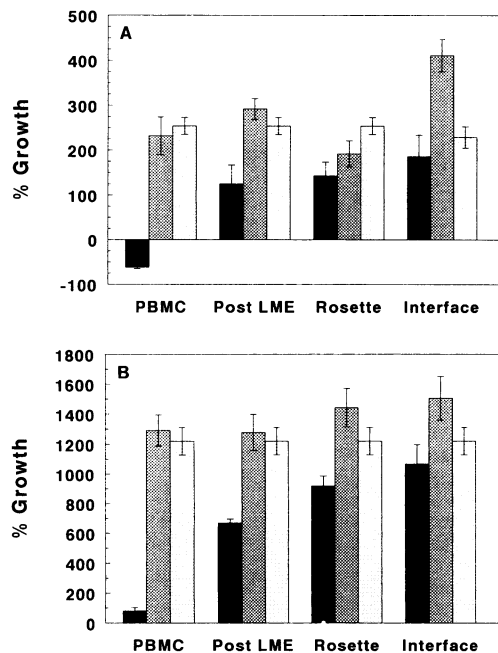**Opsonic requirements for antifungal activity.** NK cells and

FIG. 5. Effect of treatment with LME and enrichment for B cells on antifungal activity of PBMC. PBMC were either left untreated (PBMC) or incubated with 5 mM LME for 35 min (post LME). Some of the LME-treated cells were then further separated into fractions which rosetted SE (rosette, enriched for T cells) or did not rosette SE (interface, enriched for B cells). The efficiency of the procedure is shown in Table 3. C. neoformans was then added to wells containing 1 × 10^6 (solid bars), 2 × 10^5 (cross-hatched bars), or no (stippled bars) effector cells. After 24 h, fungal growth was assayed. Two cryptococcal strains were tested, B3501 (A) and 145 (B). Data represent the means ± SEM for three experiments performed in triplicate. P < 0.0001 for wells containing 10^6 PBMC versus any other group. For both strains, P ≤ 0.008 for wells containing no effector cells versus wells containing 10^6 post-LME cells.
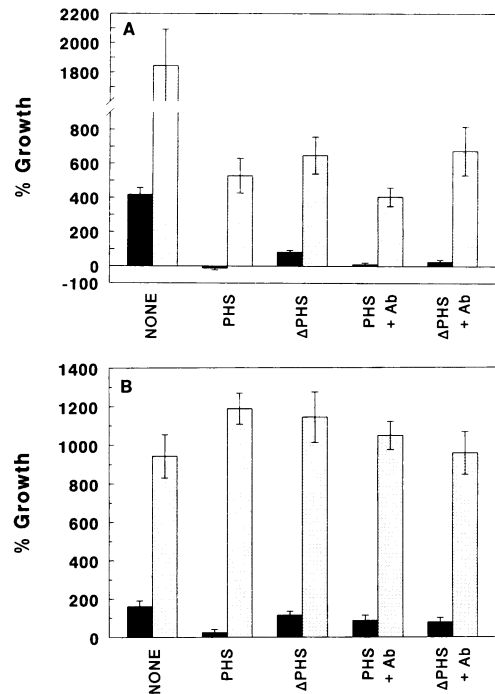

FIG. 6. Opsonic requirements for fungistasis. C. neoformans was added to wells containing 1 × 10^6 NWNA PBMC (solid bars) or no cells (stippled bars) in the presence of RPMI 1640 containing no opsonins (none); 10% PHS and 10% AB serum (PHS); 10% heat-inactivated PHS and 10% heat-inactivated AB serum (ΔPHS); 10% PHS, 10% AB serum, and rabbit anticapsular antibody (PHS + Ab); or 10% heat-inactivated PHS, 10% heat-inactivated AB serum, and rabbit anticapsular antibody (ΔPHS + Ab). Two fungal strains were tested, B3501 (A) and 145 (B). Data represent the means ± SEM for three experiments performed in triplicate. For all groups of opsonins, P < 0.0001 for wells containing NWNA PBMC versus wells containing no cells.

perhaps some T cells possess receptors for complement and Ig (36, 42). Moreover, for many cell types, opsonization with complement and/or antibody is required for immune recognition of encapsulated C. neoformans (17, 25). Therefore, we next studied the effect that various opsonic conditions would have on the ability of NWNA PBMC to inhibit the growth of two strains of C. neoformans (Fig. 6). Compared with growth inhibition in wells containing PHS alone, the addition of serotype-specific rabbit polyclonal anticryptococcal antibody and heat inactivation of serum had little or no effect on growth inhibition. For strain 145, there was a slight increase in growth when serum and antibody were omitted from the system altogether. For strain B3501, the effects of the omission of opsonins were difficult to gauge because, consistent with previous reports (13), fungal growth was increased more than threefold in wells lacking serum compared with growth in wells containing serum. Nevertheless, in the absence of opsonins, growth was 77% lower in wells containing NWNA PBMC than in wells lacking NWNA PBMC.

Activity of supernatants and cell lysates. To explore the possibility that the growth inhibition observed in the above experiments was secondary either to depletion of nutrients from the medium or to release of a preformed antifungal substance, the activity of supernatants and cell lysates derived from NWNA PBMC was assayed. Three separate
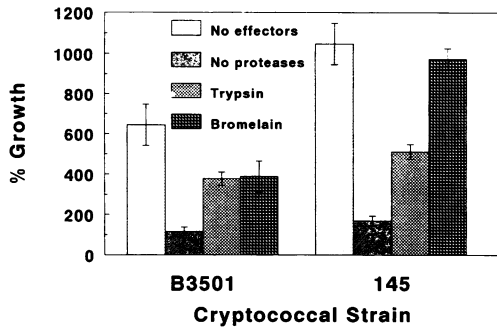
FIG. 7. Effect of receptor cleavage on fungistasis. *C. neoformans* was added to wells containing no effector cells, NWNA PBMC preincubated without proteases, or NWNA PBMC preincubated with trypsin or bromelain. Data represent the means ± SEM for three experiments performed in triplicate. For strain B3501, $P \leq$ 0.002 for no proteases versus any other group. For strain 145, $P <$ 0.0001 for no proteases versus any other group.

experiments were performed with strains B3501 and 145. Compared with cryptococcal growth in wells containing medium alone, neither supernatants (stimulated or unstimulated) nor lysates had growth-inhibitory activity (data not shown).

Effect of proteases. We next examined whether the anticryptococcal activity of NWNA PBMC would be affected by treatment with bromelain or trypsin, two proteases known to cleave some (but not all) lymphocytic receptors (8, 25). Preliminary experiments with flow cytometry established the effectiveness of protease treatment: trypsinization of NWNA PBMC diminished the expression of CD4, CD8, and CD56 by >90% while leaving expression of CD3 unaffected. After bromelain treatment, lymphocytic expression of CD8 and CD56 was reduced by >90%, whereas expression of CD3 and CD4 was unchanged. Treatment of NWNA PBMC with either protease significantly and profoundly diminished the capacity of those cells to inhibit the growth of cryptococcal strains B3501 and 145 (Fig. 7). The viability of lymphocytes 24 h after protease treatment was 93% ± 1%, 89% ± 4% and 60% ± 7% (mean ± SEM for three separate experiments) for untreated, trypsin-treated, and bromelain-treated cells, respectively.

Nomarski DIC microscopy. Lymphocytes enriched for CD4⁺, CD8⁺, or CD16/56⁺ cells by positive selection were incubated with *C. neoformans* for 2 h in medium containing 10% PHS and then fixed by immersion in methanol at −20°C for 20 s followed by three dips in acetone at −20°C. Cells were observed at 1,000× magnification by Nomarski DIC microscopy. Granules and cell boundaries are particularly well visualized by this technique. Conjugates, which were seen with all three cell types, consisted mostly of one to three lymphocytes in intimate contact with *C. neoformans* (Fig. 8). As would be expected for CD4⁺ lymphocytes, these cells were relatively devoid of granules. In contrast, conjugates between CD8⁺ or CD16/56⁺ lymphocytes and *C. neoformans* tended to be composed of granule-containing lymphocytes. For conjugates containing granular lymphocytes, the granules were usually found concentrated on or in proximity to the capsular surface of *C. neoformans*. In one representative experiment performed in duplicate, 21, 9, and 13% of CD4⁺, CD8⁺, and CD16/56⁺ lymphocytes, respectively, were conjugated with one or more *C. neoformans* cells. Actual phagocytosis (internalization) of organisms was

never seen; rather, the lymphocytic membrane could be seen in intimate contact with the capsular surface of the fungus.

Videomicroscopy. In the final set of experiments, NWNA PBMC incubated at 37°C with *C. neoformans* in the presence of 10% PHS were observed by videomicroscopy. As in previous experiments with IL-2-activated lymphocytes (22), NWNA PBMC could be seen making contact with and spreading over *C. neoformans*, often "dragging" the yeast cells across the microscope field. In many cases, several lymphocytes were observed making simultaneous or sequential contacts with an individual yeast cell. Once formed, conjugates persisted for anywhere from several minutes to more than 1 h. Phagocytosis was not observed.

DISCUSSION

The data presented here demonstrate the competence of freshly isolated human peripheral blood lymphocytes to directly inhibit the growth of *C. neoformans* in the absence of MHC restriction. By four different techniques of lymphocyte purification, CD4⁺ lymphocytes, CD8⁺ lymphocytes, and CD16/56⁺ NK cells (but not B cells) were shown to have an intrinsic ability to bind to *C. neoformans* and mediate fungistasis.

In addition to our previous data showing fungistasis mediated by IL-2-activated human T cells (22), other investigators have demonstrated direct MHC-unrestricted "promiscuity" of T cells against tumor, bacterial, and parasitic targets (16, 26, 27, 35, 40). Murphy and colleagues have demonstrated binding, growth inhibition, and killing of *C. neoformans* by murine NK cells (10, 31, 32), and in agreement with our studies, recently presented data extending these findings to human NK and T lymphocytes (30). However, two other laboratories failed to demonstrate cryptococcal fungistasis mediated by human NK or T-cell populations in the absence of anticryptococcal antibody (4, 28). The reasons for these interlaboratory disparities are not entirely clear but could relate to differences in cell densities or culture conditions. In this regard, the two laboratories demonstrating human lymphocyte-mediated fungistasis used 10⁶ effector cells per well (present study and reference 30), whereas a lower cell density was used by the other laboratories (4, 28). The interlaboratory disparities are not a result of strain variation, since lymphocyte-mediated inhibition of strain 145 occurred in our studies but not those of Miller et al. (28).

Our findings that lymphocytes treated with trypsin or bromelain had markedly impaired fungistatic capacity suggest that specific receptor-ligand interactions are necessary prerequisites for fungistasis. Since numerous receptors are cleaved by these two proteases (8, 25), future studies will be needed to determine the specific receptor(s) on lymphocytes that is responsible for this interaction. In addition to receptor cleavage, some of the inhibitory effects of bromelain (but not trypsin) may have been secondary to the decreased viability of lymphocytes treated with that protease. Our data showing that both T cells and NK cells are capable of mediating fungistasis suggest that a receptor common to both of these cell types is likely to be essential for recognition or signal transduction. As in our previous studies with IL-2-activated lymphocytes (22), complement and Ig did not appear to be essential, since fungistasis was still obtained even when serum was heat inactivated or omitted from the system entirely.

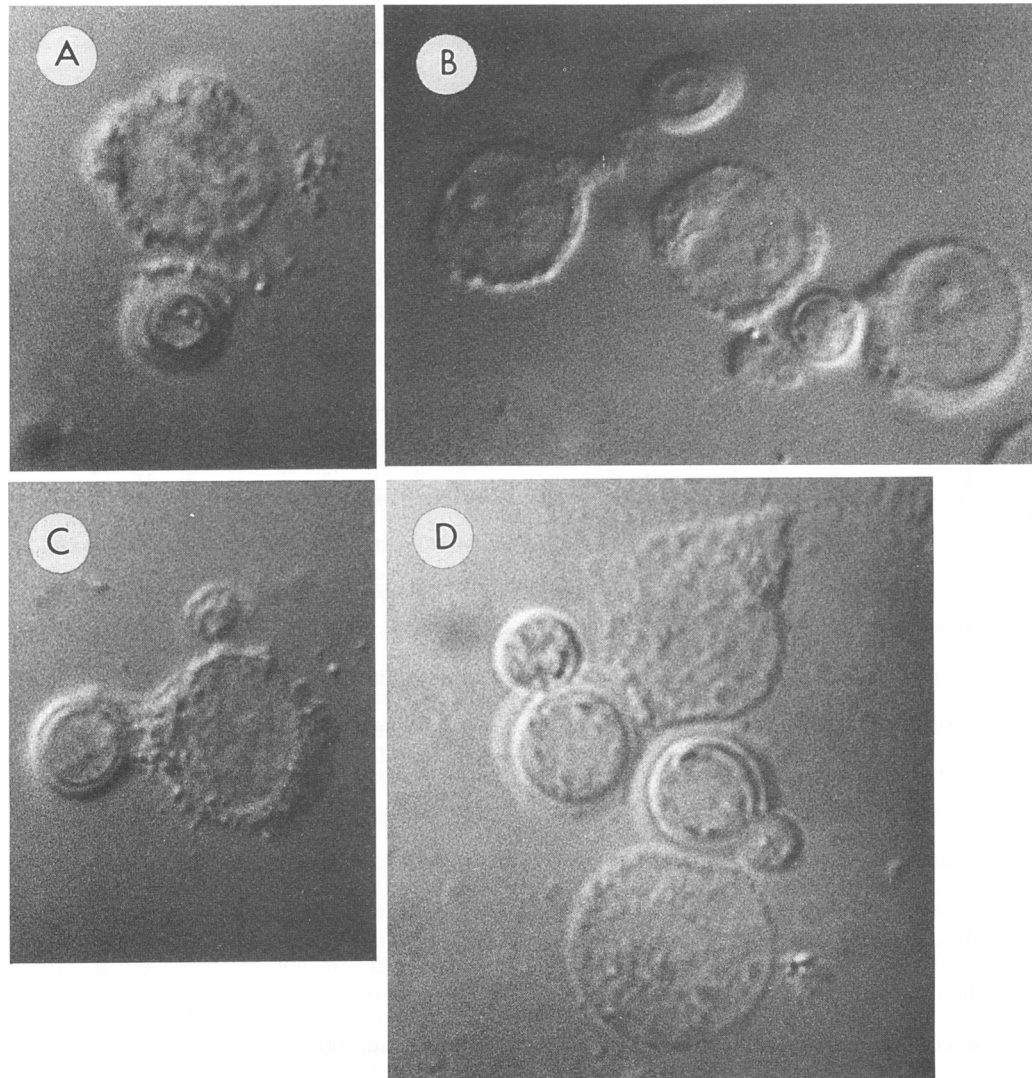NK cells and cytotoxic T lymphocytes have preformed

FIG. 8. Nomarski DIC microscopy demonstrating conjugate formation between peripheral blood lymphocyte subsets and *C. neoformans*. CD8$^+$ (A and B) CD16/56$^+$ (C), and CD4$^+$ (D) lymphocytes were isolated by positive selection of NWNA PBMC as described in Materials and Methods. The *C. neoformans* are easily identifiable by their thick cell walls and surrounding capsules. Note the intimate contact between the effector and target cells (in particular, see panel C). For many of the conjugates, granules from effector cells can be seen concentrated on or near the fungal capsular surface (in particular, see panel A). All photomicrographs are at the same magnification.

granules that can be discharged after exposure to target cells. Granule exocytosis releases preformed substances with potential effector activity, including perforin, serine esterases (granzymes), and chondroitin sulfate (36). Observations of lymphocyte-*C. neoformans* interactions under Nomarski DIC microscopy demonstrate that granule exocytosis occurs during conjugate formation. Moreover, monensin, an inhibitor of granule secretion, partially inhibits fungistasis (21). However, there is strong evidence that certain tumor targets can be lysed by NK cells and cytotoxic T lymphocytes in the absence of granule secretion (2, 7, 41). Our demonstration that CD4$^+$ lymphocytes, most of which are relatively agranular, can mediate binding and fungistasis suggests that granule-independent mechanisms of fungistasis are also operative. In support of this concept, after fractionation of rat LGL tumor cells, anticryptococcal activity was

found not only in the granule fraction but also in three cytoplasmic fractions (9).

Lymphocyte supernatants did not have measurable antifungal activity in our experiments. These data, together with those from the protease experiments, effectively rule out the possibility that lymphocyte consumption of a factor(s) essential to fungal growth was responsible for fungistasis. The inability of concentrated lymphocyte lysates to effect antifungal activity suggests that the mediator(s) of fungistasis is (i) generated after activation, (ii) short lived, and/or (iii) preferentially active in the local environment created in the course of conjugate formation.

Because of the nature of the assay used, it is difficult to determine whether, in addition to mediating fungistasis, the lymphocytes killed some of the fungal inoculum. This is because over the 24 h of incubation, some of the fungi may

have reproduced while others were killed. For many of the experiments with cryptococcal strain B3501 (but not strain 145), there was a decrease in CFU over the course of the incubation, indicating that some fungicidal activity had occurred. When the incubation period was shortened to 2 h, significant fungicidal activity was not observed (21).

It remains to be determined whether our findings have in vivo correlates. Lymphocytes are commonly found in proximity to extracellular *C. neoformans* in lesions in immunocompetent patients recovering from cryptococcosis (1, 21) and in normal mice experimentally inoculated with *C. neoformans* (11). Although a relatively high effector-to-target cell ratio was required in order to see maximal activity in our system, specifically recruited lymphocytes functioning in a physiological milieu in vivo may have activity at lower effector-to-target cell ratio. Moreover, lymphocytes may act synergistically with neutrophils, monocytes, and/or macrophages (cell types known to have antifungal activity [6, 18, 20, 23, 24, 28]) to contain the fungal burden. Finally, although the studies reported here focused on fungistasis, ongoing studies in our laboratory are examining the possibility that lymphocytes directly stimulated by *C. neoformans* may also participate in host defenses by releasing cytokines or other modulators of the inflammatory response.

ACKNOWLEDGMENTS

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