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Human Natural Killer Cells Do Not Inhibit Growth of *Cryptococcus neoformans* in the Absence of Antibody

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The interaction between human natural killer (NK) cells and yeast cells of *Cryptococcus neoformans* was investigated because experiments in mice indicated that NK cells inhibited the growth of *C. neoformans*. Strains of *C. neoformans* serotype A that differed in both resistance to alveolar macrophages and the size and composition of their capsules were evaluated. Human NK cells, which were isolated from normal peripheral blood, were activated by preincubation with interleukin-2 and alpha interferon to generate lymphokine-activated killer (LAK) cells. Yeast cells of *C. neoformans* were incubated with effector cells (NK or LAK cells); and inhibition of yeast cell growth was measured at 4, 8, and 24 h by comparing quantitative plate counts with controls consisting of yeasts in the absence of effector cells. The cytolytic activity of effector cells against target cells was confirmed by the release of radiolabel from ^{51}Cr-labeled K-562 tumor cells. Neither NK nor LAK cells inhibited the growth of 13 strains of *C. neoformans* at effector to target cell ratios of as high as 500:1. Monocytes, which were isolated from the same populations of leukocytes as the NK cells, inhibited the growth of two strains of *C. neoformans* at effector to target cell ratios of 100:1 (92 and 46% inhibition), 50:1 (87 and 17%), and 1:1 (49 and 0%). NK cells could inhibit the growth of *C. neoformans* by an antibody-dependent cellular cytotoxicity mechanism in the presence of rabbit anticryptococcal antiserum at dilutions up to 1:4,000. Purified capsular polysaccharide of *C. neoformans* had no effect on the viability or tumoricidal activity of NK or LAK cells. These data suggest that human NK and LAK cells are not impaired by *C. neoformans*, and in the absence of antibody, which is rarely detectable in patients, they afford much less protection against *C. neoformans* than monocytes do.

Cryptococcus neoformans is a significant cause of opportunistic infection in patients with impaired cell-mediated immunity (20, 21). Cryptococcal meningitis occurs more frequently in patients who have received corticosteroids, patients with hematologic malignancies, and patients with acquired immune deficiency syndrome (AIDS), in whom the incidence of cryptococcosis is estimated at 10 to 15% (9, 16).

Clinical and experimental evidence indicates that the crucial host defense mechanisms against cryptococcosis involve T-cell responses and the activities of phagocytes (2, 3, 6, 7, 18, 20, 33). However, recent murine studies suggest that natural killer (NK) cells may also represent an important mechanism of resistance against *C. neoformans* (24, 25).

Murine NK cells are able to inhibit the growth of yeast cells of *C. neoformans*, both in vitro (24, 25) and in vivo (13, 19), and the inhibition is enhanced by anticryptococcal antibody (25). Murine NK cells also inhibit the growth of other fungi, including *Paracoccidioides brasiliensis* (15) and *Candida albicans* (1). However, human NK cells do not inhibit *C. albicans* (34).

The purpose of this study was to evaluate the effectiveness of human NK cells and lymphokine-activated killer (LAK) cells against *C. neoformans*. Strains of *C. neoformans* that differed in several defined biological and biochemical properties were compared for differences in their susceptibilities to inhibition of growth by resting NK and LAK cells. In addition, strains of *C. neoformans* that were recently isolated from patients with and without AIDS were compared for their susceptibilities to LAK cells, growth rates, and sizes of their capsules and cells. The growth inhibitory activity of three effector cells—resting NK cells, LAK cells, and monocytes—was compared, and the influence of rabbit anti-*C. neoformans* antibody on NK cells was evaluated. In addition, the effect of yeast cells of *C. neoformans* and purified capsular polysaccharide (CPS) (29) on the tumoricidal activity of NK cells was tested.

MATERIALS AND METHODS

Strains of *C. neoformans*. Seven previously characterized strains of *C. neoformans* serotype A and six recent clinical isolates of *C. neoformans* were studied. The strains designated 6, 15, 98, 110, and 145 are stable variants of serotype A that differ in (i) the sizes of their capsules; (ii) the degree to which they are phagocytized and killed by alveolar macrophages (2, 3); and (iii) the relative molecular size, composition, and binding capacity of their CPSs (28, 29). Strain 184, also serotype A, was generously donated by Juneann W. Murphy (22). The capsule-free mutant, strain 602, was a gift from Thomas R. Kozel (17). The six recent clinical isolates were obtained from the Clinical Mycology and Mycobacteriology Section of the Clinical Microbiology Laboratory at Duke Hospital and designated with the accession numbers 1188, 1218, 1458, 1508, 1948, and 1988; these strains were evaluated within 6 months or less from the time of their recovery from patient specimens. The underlying diseases of the patients and the specimens from which these strains were isolated, the sizes of the yeast cells and capsules, and the growth rates of these strains are presented in Table 1.

All yeasts were maintained at 37°C on slants composed of 2% glucose and 1% yeast extract (Difco Laboratories, Detroit, Mich.) (GYE) with 2% agar and were transferred at biweekly intervals. The diameters of the yeast cells and capsules were determined by microscopic examination of India ink preparations. Each isolate was cultured at 37°C for 3 days on GYE slants, and at least two investigators, on

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TABLE 1. Strains of *C. neoformans* used in this study

Strain[a]	Source or reference[b]	Capsule width (μm)[c]	Cell diam (μm)[c]	Growth rate (h)[d]
6	2, 29	>3.5	4.4 ± 0.8	ND
15	2, 29	<1.0	5.0 ± 0.8	6.0
98	2, 29	2.0 ± 0.8	4.0 ± 0.6	6.7
110	2, 29	2.4 ± 0.8	4.9 ± 1.0	5.2
145	2, 29	1.7 ± 0.4	5.1 ± 0.8	7.0
184	23, 24	2.6 ± 1.5	4.7 ± 1.0	ND
1988	Blood, vasculitis's	2.0 ± 0.9	5.0 ± 0.9	5.3
1218	Bronchial wash, Wegener disease	<1.0	4.2 ± 0.8	6.0
1458	Blood, Hodgkin's disease	1.6 ± 0.8	5.2 ± 1.0	ND
1508	CSF, AIDS	<1.0	5.2 ± 0.8	ND
1948	CSF, AIDS	2.3 ± 1.0	4.2 ± 0.8	6.0
1188	CSF, AIDS	<1.0	4.9 ± 1.0	5.2
602	17	None	3.8 ± 0.6	ND

[a] Strains 6, 15, 98, 110, and 145 have been studied previously and differ in the composition of their capsular polysaccharides (28, 29) and resistance to phagocytosis and killing by alveolar macrophages (2, 3). Strain 184 was inhibited by murine NK cells (23–25). Strains 1988, 1218, 1458, 1508, 1948, and 1188 were isolated during 1988 from patients at Duke Hospital. Strain 602 is a capsule-free mutant (17).

[b] For the recent clinical isolates, the patient specimen and underlying disease are indicated. CSF, Cerebrospinal fluid.

[c] After growth for 3 days at 37°C, samples of each strain were mounted in India ink, and the dimensions of 50 yeast cells were measured. Capsule width is the thickness of the capsule; measurement of the cell diameter excludes the capsule. Values represent at least three sets of measurements ± standard deviation.

[d] Yeasts were grown in broth medium at 37°C on a gyratory shaker at 150 rpm, and at 6-h intervals, the CFU per milliliter was determined by quantitative plate counts. Each value is the mean doubling time computed during exponential growth. ND, Not done.

separate occasions, measured the dimensions of 50 cells. To determine the growth rates, yeast cells were cultured for 3 days at 37°C on GYE slants, and dilute suspensions in sterile water were prepared. One milliliter of this suspension was used to establish liquid cultures in 500-ml Erlenmeyer flasks containing 200 ml of phosphate-buffered glutamine-glycine-asparagine (GGA-B) broth (29). These cultures were incubated at 37°C on a gyratory shaker at 150 rpm. Every 6 h, each culture was sampled. The number of yeast cells was estimated by microscopic counts in a hemacytometer chamber, and the viable census was determined by quantitative plating of appropriate dilutions of each sample. At each time point, duplicate dilutions and three pour plates of each dilution were prepared. Pour plates were seeded with 1.0 ml of each dilution and mixed with molten GYE cooled to 48°C. The plates were incubated for 3 days, and the number of CFU per plate was counted. The doubling time of each strain was calculated.

Isolation and activation of NK cells. NK cells were isolated from the peripheral blood of healthy human donors, as previously described (30). Venous blood was collected in 20 U of heparin per ml and centrifuged at 500 × *g* for 30 min, and the resulting buffy coat was removed. Leukocytes were suspended in medium that was composed of RPMI 1640 (GIBCO Laboratories, Grand Island, N.Y.) and supplemented with L-glutamine, 25 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) buffer, 100 U of penicillin per ml, and 100 μg of streptomycin per ml (RPMI). The cells were layered on a discontinuous gradient of Ficoll-Hypaque (LSM; Organon Teknika, Durham, N.C.) and centrifuged at 500 × *g* for 30 min. The cells at the resulting interface were removed, washed, and suspended in RPMI with 10% (vol/vol) heat-inactivated fetal bovine serum (GIBCO) (RPMI-FBS). T cells were removed by erythrocyte-rosette depletion with 2-aminoethylisothiouraniumbromide hydrobromide-modified sheep erythrocytes (27). The remaining cells were washed and suspended in RPMI-FBS. Adherent cells were depleted by incubation in 75-cm^2 plastic culture flasks (Corning Glass Works, Corning, N.Y.) for 60 min at 37°C under 5% CO_2. The flasks were washed three times with warm phosphate-buffered saline at pH 7.4 to collect nonadherent cells, which were subsequently fractionated on a two-step discontinuous Percoll (Pharmacia Fine Chemicals, Piscataway, N.J.) gradient. The upper Percoll layer had a density of 1.057 g/ml and a refractive index of 1.3420 (at 23°C); the density of the lower layer was 1.064 g/ml and the refractive index was 1.3433. Gradients were centrifuged at 500 × *g* for 30 min, and the NK-enriched population of cells was collected from the interface of the two Percoll layers. These resting NK cells were resuspended, washed, counted in a hemacytometer, and adjusted to 10^6 cells per ml of RPMI-FBS. NK preparations typically had the following phenotypes: >60% Leu-7 (CD57) positive, >75% Leu-19 (CD56) positive, >85% OKM1 (CD11b) positive, >60% Leu-11b (CD16) positive, <1% MO2 (CDw14) positive, <5% OKT3 (CD3) positive, <15% B1 (CD20) positive, and <20% 4F2 (activation antigen) positive (data not shown) (30).

To activate the resting NK cells, 50 U of interleukin-2 per ml (8) and 50 U of alpha interferon per ml (31) were added to 10^6 NK cells per ml of RPMI-FBS, and the cells were incubated overnight at 37°C in 5% CO_2 (and 95% air). These LAK cells were then washed and suspended at 10^6/ml in RPMI-FBS. The phenotypes of these activated NK cells were indistinguishable from those cited above for the resting, endogenous NK cells, with the notable exception of 4F2 antigen expression. LAK cells were >80% 4F2 positive (data not shown).

Tumoricidal activity. To quantify the cytolytic activity of NK and LAK cells, target cells of the tumor line K-562 were used in a standard 4-h ^{51}Cr-release assay (14). Briefly, 10^6 K-562 cells were radiolabeled by incubation for 2 h at 37°C in 100 μl of RPMI containing 100 μCi of $Na_2{}^{51}CrO_4$ (200 to 500 μCi/mg of ^{51}Cr; Dupont, NEN Research Products, Boston, Mass.). The cells were washed three times in RPMI-FBS and suspended at 10^5/ml of RPMI-FBS. NK or LAK effector cells were added to 100 μl of K-562 target cells in the round-bottom wells of microtiter plates (3799; Costar, Cam-

bridge, Mass.), and RPMI-FBS was added to yield a final volume of 200 μl per well and effector:target cell (E:T) ratios of 2.5:1, 5:1, and 10:1. Each combination was established in triplicate wells, the plates were incubated for 4 h at 37°C in 5% CO_2 and centrifuged, and the radioactivity in the supernatants was measured in a gamma counter (Biogamma; Beckman Instruments, Inc., Fullerton, Calif.). The percent cytolysis was calculated as follows: percent cytolysis = 100 × [(experimental cpm − spontaneous cpm)/(mean maximal cpm − spontaneous cpm)]. Experimental counts per minute represents the mean counts per minute of radioactivity in wells containing both effector and radiolabeled target cells. Spontaneous counts per minute is the counts per minute released by target tumor cells that were incubated in RPMI alone; maximal counts per minute is the mean counts per minute released by target cells that were treated with 0.25% (vol/vol) Triton X-100 (Sigma Chemical Co., St. Louis, Mo.).

Inhibition of growth of *C. neoformans* by NK or LAK cells. Anticryptococcal activity was evaluated by comparing the percentage of viable yeasts incubated in the presence of effector cells (resting NK or LAK cells) with control growth in the absence of effector cells. The viable yeast census was determined by a quantitative pour plate assay. After growth for 3 days on GYE slants at 37°C, yeast cells of *C. neoformans* were harvested, counted in a hemacytometer chamber, and diluted in RPMI-FBS to 10^4 yeast cells per ml. Effector cells (resting NK or LAK cells) were suspended in RPMI-FBS at 10^6 cells per ml, and 100 μl of this suspension was placed in the conical wells of microtiter plates (220-25; Cooke, Alexandria, Va.). To achieve E:T ratios of 500:1 and 100:1, 20- or 100-μl portions of the yeast suspension were added to respective wells, and RPMI-FBS was added, when necessary, to bring the final volume in all wells to 200 μl. Control wells contained only yeast cells and RPMI-FBS. Plates were incubated for 4, 8, and 24 h at 37°C under 5% CO_2. Triplicate wells were set up for each ratio, control, and incubation period tested. At each time period, the appropriate wells were washed three times in sterile water, and the washings were pooled, diluted, and thoroughly mixed. The contents of each well were plated at two dilutions. Samples of 200 μl to 1.0 ml, depending on the incubation time and strain, were placed in sterile plastic petri plates. Molten GYE at 48°C was poured over the samples, and the plates were mixed, allowed to solidify, and incubated at 37°C. The viability of the effector cells, as determined by the exclusion of trypan blue, was >90% at each incubation time, in the presence or absence of yeast cells. The CFU of *C. neoformans* was counted after 3 days of incubation, and the percentage of control growth was calculated as follows: 100 × (mean experimental CFU/mean control CFU). The mean CFU of each experimental and control value was calculated from six plates: three plates of a relatively low dilution that usually resulted in 80 to 180 CFU per plate and three plates of a higher dilution that usually yielded 25 to 80 CFU per plate. The mean CFU of each triplicate was multiplied by the dilution factor, and the average of the two computations produced the final mean CFU. The standard deviation for each mean rarely exceeded 10% and was always less than 15%.

Inhibition of growth of *C. neoformans* by monocytes. Adherent cells from the NK separation were washed twice with warm phosphate-buffered saline and mechanically removed with a rubber policeman. Viability, as determined with trypan blue, ranged between 80 and 90%, and >95% of the cells were monocytes based on a bulk population phenotype that was >95% OKM1 positive and <1% positive for the Leu-7, Leu-19, OKT3, and B1 markers (data not shown). The cells were suspended in RPMI-FBS at 3×10^6 viable monocytes per ml, and 100 μl was added to flat-bottom wells of microtiter plates (3596; Costar). The plates were incubated for 60 min at 37°C under 5% CO_2, and the medium was removed and replaced with 100 μl of RPMI with 10% normal human serum, which was collected from healthy volunteers. Yeasts were harvested as described above and suspended in RPMI at 3×10^5 yeast cells per ml. Samples of 10 or 20 μl of this suspension were added to the wells to yield monocyte to yeast (E:T) ratios of 100:1 and 50:1, respectively. An E:T ratio of 1:1 was achieved by adding 100 μl of yeasts at 3×10^6/ml of RPMI with 10% normal human serum. After incubation for 4 h at 37°C in 5% CO_2, 100 μl of 0.25% (vol/vol) Triton X-100 was added to all wells to lyse the monocytes and release phagocytized yeast cells, and the plates were reincubated for 15 min. Growth at each ratio was assessed in triplicate and compared with growth in corresponding control wells containing only yeast cells in RPMI with 10% normal human serum. The wells were washed three times with sterile water, the washes were pooled and serially diluted, and portions of the final dilution were placed in petri plates to which molten GYE was added as described above. Microscopic examination of wells and wash fluids revealed that the monocytes were completely lysed by Triton X-100. Residual cell fragments were not visible in the wells or the wash fluids. After 3 days of incubation at 37°C, the number of CFU was enumerated and the percentage of control growth was calculated as described above. Preliminary experiments confirmed that, under these conditions, Triton X-100 had no effect on the viability or clumping of the yeast cells.

Effect of the *C. neoformans* CPS on NK and LAK cells. To determine whether the CPS of *C. neoformans* had any effect on the effector cells, resting NK and LAK cells were exposed to purified CPS from strain 110 (29) and tested for tumoricidal activity. CPS was dissolved in RPMI at 1,000 μg/ml. Resting NK or LAK cells were suspended at 10^6 cells per ml in a final volume of 300 μl of RPMI containing 0, 100, 200, or 400 μg of CPS per ml. The tubes were incubated for 18 h at 37°C under 5% CO_2. Ten minutes before the 18-h incubation period ended, CPS was added at the same concentrations to a set of effector cells that had been incubated without CPS. The effector cells from both 0- and 18-h incubations in CPS were then tested for tumoricidal activity as described above at E:T ratios of 10:1 and 2.5:1. Triplicate wells were assayed for each E:T ratio, concentration of CPS, and effector cell that was tested.

Effect of anti-*C. neoformans* antiserum on the inhibition of growth of *C. neoformans* by NK cells. The participation of NK cells in antibody-dependent cellular cytotoxicity against *C. neoformans* was evaluated. Rabbit anti-*C. neoformans* antisera, which were generously donated by Errol Reiss, were diluted in RPMI-FBS and added to the NK inhibition assay described above at final concentrations of 1:1,000, 1:2,000, and 1:4,000. The dilutions of antisera were added in volumes of 50 μl to wells containing NK cells, and then yeast cells of strain 184 were added. The assay of antibody-dependent cellular cytotoxicity was tested at NK to yeast cell ratios of 500:1, 50:1, and 10:1. For the 50:1 ratio, yeasts cells were suspended at 10^5 yeasts per ml in RPMI-FBS, and 20 μl was added to the appropriate wells; for the 10:1 ratio, 10 μl of a suspension of 10^6 yeasts per ml was added. RPMI-FBS was added to adjust the final volume to 200 μl per well. Controls included wells that contained yeast and NK cells that were

incubated with nonimmune rabbit serum at concentrations similar to that of the antiserum. To test for any effect of serum on the viability counts, yeast cells were incubated in control wells with RPMI-FBS but no NK cells and appropriate amounts of normal rabbit serum or antiserum. All test and control wells were set up in triplicate and incubated for 4 or 8 h at 37°C under 5% CO_2. As described above, the contents of each well were removed and diluted twice, each dilution was plated in GYE, the plates were incubated at 37°C, and the number of CFU per well was determined. Test results are expressed as the percentage of yeast cell control growth in the absence of NK cells.

Statistical analysis. The Student *t* test for paired means was applied to the growth inhibition data. The experimental mean was compared with the control mean for each experiment.

RESULTS

Strains of *C. neoformans*. Since variations in the growth rates of the different strains of *C. neoformans* could affect the assays of growth inhibition, growth curves were generated for most of the strains, and the doubling times were calculated during logarithmic growth. In either GGA-B or RPMI-FBS medium, the mean doubling time and standard deviation was 5.93 ± 0.68 h and ranged from 5.2 to 7.0 h (Table 1). In RPMI-FBS, the lag phases of the recent isolates (strains 1988, 1218, 1458, 1508, 1948, and 1188) were shorter than those of the other strains. Since growth was minimal during the initial 4-h sampling time, a reduction in the viable census at this time was assumed to represent killing.

On primary isolation, yeast cells from strains of *C. neoformans* isolated from patients with AIDS have been reported to be smaller than yeast cells from other patients (4, 5). As indicated in Table 1, differences in the capsule and cell sizes of these three isolates did not correlate with their source.

Tumoricidal activity of NK and LAK cells. The cytotoxicities of effector cells, resting NK and LAK cells, were compared against the prototypic target cells for human NK cells, the K-562 erythroleukemia cell line (26). Effector cells were harvested from the fraction of cells with an intermediate density in Percoll; this fraction has been shown to exhibit 80 to 90% of the NK cell cytolytic potential, as assayed by a 4-h chromium release assay (14). In each experiment in which the activities of NK and LAK cells were compared, the same population of cells was used. Resting NK cells were incubated overnight in RPMI-FBS alone, and LAK cells were generated by incubating NK cells with interleukin-2 and alpha interferon. The following day, the tumoricidal activity of resting NK and LAK cells was evaluated at E:T ratios of 2.5:1, 5:1, and 10:1.

As indicated in Table 2, resting NK cells obtained from the intermediate Percoll fraction possessed appreciable tumoricidal activity at all E:T ratios. The LAK cells that were generated from these NK cells displayed significantly increased cytolytic activity. The activity of the LAK cells exceeded that of the resting NK cells by factors of 3.6, 1.8, and 1.6 at E:T ratios of 2.5:1, 5:1, and 10:1, respectively.

Inhibition of growth of *C. neoformans* by NK and LAK cells. The effects of human NK and LAK cells on *C. neoformans* were tested under experimental conditions that were similar to reports of the anticryptococcal activities of murine NK cells (23, 25). Five strains of *C. neoformans* serotype A (strains 145, 110, 98, 15, and 6) that differ in their susceptibilities to phagocytosis and killing by alveolar macrophages (2, 3), as well as the sizes and chemical properties of their capsules (28, 29), were compared to detect any strain differences in resistance to growth inhibition by NK cells. Strain 184 was used in the murine NK cell studies (23, 25). The capsule-free mutant, strain 602, was also included (17, 18). Yeast (target) cells of each strain were tested against both resting NK and LAK effector cells at E:T ratios of 500:1 and 100:1, and growth was quantified in the presence and absence of effector cells after incubation for 4, 8, and 24 h. (Preliminary experiments with NK cells showed no inhibition at E:T ratios of 10:1 and 50:1.)

The percentage of control growth of these strains in the

TABLE 2. Tumoricidal activity of NK and LAK cells against K-562 tumor cell targets

E:T ratio[a]	% Cytolysis by[b]:	
	NK[c]	LAK[c]
10:1	51 ± 5.2	80 ± 4.1
5:1	36 ± 3.3	65 ± 5.2
2.5:1	17 ± 1.2	62 ± 3.2

[a] Effector cells were NK or LAK; target cells were K-562.
[b] Each value is the mean percent cytolysis ± standard error of the mean of from three to six experiments.
[c] Student *t* test for paired means, $P < 0.01$, comparing NK and LAK values at all three E:T ratios.

TABLE 3. Lack of inhibition of growth of *C. neoformans* by human NK and LAK cells

Strain	Time (h)	% of control growth of strain at indicated E:T ratio[a]:			
		Resting NK cell		LAK cell	
		500:1	100:1	500:1	100:1
602	4	108	115	118	116
	8	112	112	160	130
	24	105	103	109	104
145	4	113	107	105	108
	8	93	107	110	104
	24	117	115	103	118
145	4	118	99	95	104
	8	105	96	90	97
	24	84	112	101	109
110	4	102	101	112	107
	8	104	100	107	105
	24	95	97	119	100
98	4	109	117	105	100
	8	111	105	100	101
	24	92	108	119	108
15	4	105	110	102	100
	8	86	106	102	105
	24	99	119	110	101
6	4	95	105	98	97
	8	101	111	96	104
	24	107	111	110	106

[a] Values are the percent mean experimental CFU per well divided by mean control CFU per well without NK or LAK cells; each value represents two or more experiments, with at least 12 plates per value (see text). $P > 0.05$ for all values, comparing the experimental mean with the control mean. E:T is the ratio of NK or LAK cells to yeast cells of *C. neoformans*.

TABLE 4. Lack of inhibition of growth of recent clinical isolates of *C. neoformans* by human LAK cells

Strain	Time (h)	% of control growth at E:T ratio[a]:	
		500:1	100:1
1988	4	98	102
	8	104	106
	24	103	109
1218	4	98	102
	8	105	104
	24	107	109
1458	4	98	98
	8	102	99
	24	104	104
1948	4	102	103
	8	101	100
	24	100	107
1188	4	101	110
	8	104	99
	24	100	107
1508	8	101	100
	24	104	103

[a] Values are the percent mean experimental CFU per well divided by mean control CFU per well without LAK cells; each value represents two or more experiments, with at least 12 plates per value; $P > 0.05$ for all values.

presence of effector cells is indicated in Table 3. Human NK cells did not significantly inhibit or enhance the growth of any of the *C. neoformans* strains. The Student *t* test for paired means was $P > 0.05$ for each experimental mean CFU per well compared with the corresponding control CFU per well. In microtiter wells with medium alone, none of the strains multiplied appreciably by 8 h, but all were growing logarithmically at 24 h.

The six recent clinical isolates were also resistant to LAK cells, as shown in Table 4. These strains varied with source and underlying disease of the patient, as well as capsule and cell size (Table 1). These strains entered logarithmic growth between 4 and 8 h, which was earlier than that of the other strains.

NK and LAK viability was determined by trypan blue exclusion and exceeded 90% for all time periods and experiments, with or without the presence of yeast cells.

Inhibition of growth of *C. neoformans* by monocytes. To confirm that human monocytes were able to inhibit the growth of *C. neoformans* (6) and to provide a control for the assay of growth inhibition, human monocytes were recovered from the same blood samples from which NK cells were obtained. Both the encapsulated strain 98 and the capsule-free strain 602 were inhibited by monocytes, as shown in Table 5. Killing occurred at E:T ratios of 100:1 and 50:1 for both strains and at 1:1 for strain 98. The assays were highly reproducible, and growth inhibition correlated with the E:T ratios. (Triton X-100, which was added to lyse the monocytes and release phagocytized yeast cells, did not affect the viability of the yeasts, and no clumping of yeast cells occurred in experimental dilutions.) Similar to the recent report by Levitz and DiBenedetto (18), the encapsulated yeasts were inhibited to a significantly greater extent than the capsule-free strain.

Effect of CPS on NK and LAK cells. To determine whether

TABLE 5. Inhibition of growth of *C. neoformans* by monocytes

Strain	% of control growth at the following E:T ratio[a]:		
	100:1	50:1	1:1
602	54 ± 2.5	83 ± 0.5	100
98	8 ± 3.5	13 ± 2.0	51 ± 1.5

[a] Values are the percent mean experimental CFU divided by mean control CFU without monocytes ± standard error of the mean. Data represent two experiments, with 12 plates for each value. E:T is the ratio of monocytes to yeast cells of *C. neoformans*.

the tumoricidal activity of the NK and LAK cells was affected by *C. neoformans*, purified CPS from *C. neoformans* 110 (29) was added to both effector cells at final concentrations of 100, 200, and 400 μg/ml. The effector cell populations were split and incubated with CPS for either 10 min or 18 h, to discriminate between a potential binding or metabolic effect, respectively, and the cells were assayed for tumoricidal activity against radiolabeled K-562 cells at E:T ratios of 2.5:1 and 10:1. CPS had no effect on the viability of the effector cells. At all of the CPS concentrations, as well as both exposure times and E:T ratios, CPS had no significant effect—neither inhibition nor enhancement—on the tumoricidal activity of the NK or LAK cells.

Effect of anti-*C. neoformans* antibodies on the inhibition of growth of *C. neoformans* by NK cells. In the presence of rabbit anticryptococcal antiserum, human NK cells functioned as effectors in an antibody-mediated cellular cytotoxicity reaction that inhibited the growth of *C. neoformans*. The antiserum was tested at final dilutions of 1:1,000, 1:2,000, and 1:4,000, and NK to yeast cell ratios of 10:1, 50:1, and 500:1 were evaluated. Control wells contained similar mixtures of NK cells, yeasts, and nonimmune rabbit serum. After incubation for 4 or 8 h, growth of the yeast cells was quantified and compared with growth in wells lacking NK cells. At the E:T ratio of 500:1, the CFU of *C. neoformans* 184 per well was reduced to 48 to 51% of control growth in all concentrations of antiserum after 4 h and to 18 to 24% by 8 h. With the antiserum diluted 1:2,000, the numbers of viable yeasts remaining at 4 h were 65, 27, and 50% of the controls at E:T ratios of 10:1, 50:1, and 500:1, respectively. Growth of the yeast cells was not inhibited by NK cells and nonimmune rabbit serum. Yeast cells were neither clumped nor killed in the presence of either serum alone, as the number of CFU per well was similar among wells containing yeasts in antiserum, nonimmune serum, or RPMI-FBS.

DISCUSSION

Neither human resting NK nor LAK cells had any effect on the growth of *C. neoformans* in vitro. Similar, Percoll-fractionated effector cells from mice have been reported to inhibit the growth of *C. neoformans* 184, and inhibition of these yeast cells was augmented by the addition of anticryptococcal antibody (23–25).

Few differences between mouse and human NK cells have been documented. Fifty percent of human NK cells express receptors for sheep erythrocytes, whereas approximately half of mouse NK cells express Thy-1 and 20% express Lyt-1 (26). Hansson et al. (10) compared the intra- and interspecies reactivities of human and mouse NK cells against tumor cell lines; they found a preference for intraspecies recognition of tumor targets, but some mouse NK cell cytotoxicity was observed against human tumor targets.

In this investigation, the effect of human NK cells on growth of *C. neoformans* was evaluated at 4, 8, and 24 h. The 4- and 8-h time periods were selected to detect any early reduction in the inoculum before significant growth of surviving yeasts could mask inhibition. (The doubling time of all the strains tested was similar and averaged ca. 6 h.) The kinetics of tumor cell killing suggested that any growth inhibition would have been evident by 24 h. To optimize conditions for growth inhibition, ratios of NK to yeast cells of 500:1 and 100:1 were tested. Similar and smaller ratios were effective in the murine system (23, 24).

The efficacy of purified human NK and LAK cells was confirmed by quantifying tumoricidal activity against K-562 cells, the prototypic tumor target for human NK cell cytotoxicity. Both effector cells demonstrated appreciable cytotoxic activity, and treatment of the NK cells with interleukin-2 and alpha interferon significantly increased this activity (Table 2).

A number of previously characterized and recently isolated strains of *C. neoformans* were investigated to explore potential strain variation in inhibition of growth by human NK and LAK cells. The strains varied in several properties, including (i) the dimensions of the yeast cells and capsules (Table 1); (ii) the chemical composition, molecular size, and binding characteristics of their CPSs (28, 29); (iii) resistance of the cells to phagocytosis and killing by alveolar macrophages (2, 3); and (iv) their source and association with patients with AIDS (Table 1). All the strains had similar rates of growth (Table 1). None of the strains was significantly inhibited by NK or LAK cells at any of the incubation times or effector to yeast cell ratios, including strain 602, a capsule-free mutant, and strain 184, which was inhibited by murine NK cells (23, 24).

Six of the strains of *C. neoformans* were tested within 6 months of their isolation from patients and provided a comparison of strains from patients with and without AIDS (Table 1). Strains from patients with AIDS have been reported to have smaller capsules and overall sizes (4, 5), and patients with AIDS have been reported to have impaired NK cell activity (32). The strains from patients with AIDS used in this study were poorly encapsulated and relatively small in total (capsule plus cell) size, but they were not significantly different from the strains from other patients (Table 1). Although the growth rates of all the strains were similar, the six recently isolated strains had shorter lag phases than did the other strains in both GGA-B and RPMI-FBS media. These strains were equally resistant to LAK cells (Table 4).

The studies of murine NK activity found no correlation between capsule size and inhibition of growth of *C. neoformans* strains (23). Murine NK cells recognize and bind a component of *C. neoformans* that is located at the cell wall, beneath the capsule (24).

C. neoformans had no effect on the viability or tumoricidal activity of NK and LAK cells. Whole yeast cells did not reduce the viability of the effector cells during the 24-h growth inhibition assay. The CPS from strain 110 was added to NK and LAK cells at concentrations of 100, 200, or 400 μg/ml, and cytotoxicity was measured immediately or after incubation for 18 h. No inhibitory or stimulatory effect on NK or LAK activity was observed under any of the conditions tested.

The inhibition of growth of *C. neoformans* by monocytes provided a positive control for the assay of growth inhibition. Both encapsulated and capsule-free yeasts were inhibited (Table 5). The results were highly reproducible, and killing correlated with increasing ratios of monocytes to yeast cells. The percent growth inhibition was comparable to those presented in previous reports (6, 18). In confirmation of the results of Levitz and DiBenedetto (18), the capsule-free strain was more resistant than the encapsulated strain.

The enhancement of growth inhibition by NK cells in the presence of small amounts of rabbit anti-*C. neoformans* antibody was dramatic. This antiserum had a high titer of anti-CPS antibodies, as detected by indirect immunofluorescence (data not shown), which would facilitate contact between NK and yeast cells. The antibody alone had no effect on the determination of yeast growth. However, since specific antibody is rarely detectable or produced by patients with cryptococcosis (11, 12), the significance of NK-mediated, antibody-dependent cellular cytotoxicity as an important defense against cryptococcosis is questionable.

Results of this investigation suggest that human NK and LAK cells are not an important component of the natural immune response against *C. neoformans*. Murine and human NK cells exhibit significant differences in their cytotoxicity for *C. neoformans* and perhaps other microbial targets. Other peripheral host defenses, such as neutrophils, monocytes, and antibody-enhanced NK cells, are more effective anticryptococcal defenses. These defense mechanisms and strain differences in susceptibility to them are under investigation.

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