INFECTION AND IMMUNITY, Oct. 1985, p. 50–57
0019-9567/85/100050-08$02.00/0

Vol. 50, No. 1

In Vitro Binding of Natural Killer Cells to *Cryptococcus neoformans* Targets

NASRIN NABAVI AND JUNEANN W. MURPHY*

Department of Botany and Microbiology, University of Oklahoma, Norman, Oklahoma 73019

Received 8 April 1985/Accepted 17 June 1985

Nylon wool-nonadherent splenic cells from 7- to 8-week-old CBA mice were further fractionated on discontinuous Percoll gradients. Enrichment of natural killer (NK) cells in Percoll fractions 1 and 2 was confirmed by morphological examination, by immunofluorescent staining, and by assessing the cytolytic activity of each Percoll cell fraction against YAC-1 targets in the 4-h ^{51}Cr release assay. Cells isolated from each Percoll fraction were tested for growth-inhibitory activity against *Cryptococcus neoformans*, a pathogenic yeastlike organism, by using an in vitro 18-h growth inhibition assay. The results showed that NK cell enrichment was concomitant with enrichment of anti-Cryptococcus activity in Percoll fractions 1 and 2. Cells from NK cell-rich fractions formed conjugates with the mycotic targets similar to the conjugates reported in NK cell-tumor systems. In addition, the percentage of effector cell-*Cryptococcus* conjugates was directly proportional to the level of the *C. neoformans* growth-inhibitory activity of the effector cells used. Scanning electron microscopy of the effector cell-*Cryptococcus* conjugates showed direct contact between the effector cells and the cryptococcal targets. An immunolabeling method combined with scanning electron microscopy was used to demonstrate that the effector cells attached to *C. neoformans* were asialo GM_1 positive and, therefore, had NK cell characteristics.

Cryptococcus neoformans is a ubiquitous, encapsulated yeastlike organism which causes infections in humans ranging from asymtomatic pulmonary infections to fatal disseminated disease. The most severe cryptococcal disease usually occurs in immunocompromised individuals (3, 26). Since *C. neoformans* is present in nature, many people are exposed to it, but only a small number actually acquire the disease. This implies that there must be an active natural surveillance mechanism present in hosts against this organism.

Murine models have provided insights into the interaction of the resistance mechanisms of hosts against cryptococci, and by using such models, researchers have described both natural and acquired host defenses. Macrophages and polymorphonuclear leukocytes have been reported as providing innate host defenses against *C. neoformans* (1, 4, 27). Recently, data generated by Murphy and McDaniel (21) indicated that natural killer (NK) cells are potentially the third means of natural cellular resistance against this organism. The data of these authors provided for the first time substantial indirect evidence that unstimulated murine splenic cells with characteristics of NK cells can effectively inhibit the in vitro growth of *C. neoformans* (21). It was shown that the cytotoxicities of the various murine splenic effector cell populations against YAC-1 targets directly correlated with the abilities of the effector cells to inhibit cryptococcal growth. When adherent cells were removed from the effector cell pools by passage through nylon wool columns, both NK cell activity against YAC-1 targets and growth-inhibitory activity against *C. neoformans* increased. The effector cells were similar to NK cells in that they were nylon wood-nonadherent (NWN) cells without immunoglobulin or significant amounts of Thy1 or Ia antigens on their surfaces but expressed an asialo GM_1 surface marker (21). Although growth inhibition of *C. neoformans* by NK cells or NK-like cells has been demonstrated, the nature of the effector cell-*Cryptococcus* target interactions is not yet known. Therefore, we began studies directed toward elucidating the mechanisms by which NK cells affect *C. neoformans* targets. The NK cell-tumor target model appeared to be a useful guide for these investigations. In this model, NK cells kill their targets through a nonphagocytic means, by forming conjugates with their tumor targets in vitro (6, 9, 24, 25). Binding of the effector cells to the targets is a prelytic event in NK cell-tumor systems (9, 24), so one interest which we had was whether effector cell-*C. neoformans* conjugates could be observed in our system. In this report, we present data which show that NK cell-enriched populations formed conjugates with *C. neoformans* targets. Furthermore, the numbers of effector cell-*C. neoformans* conjugates were directly proportional to the levels of growth-inhibitory activity of the effector cell populations. Finally, using immunolabeling combined with scanning electron microscopy (SEM), we demonstrated that effector cells which were attached to cryptococci were asialo GM_1 positive, a characteristic of NK cells (11, 13). Considering (i) that the cell populations being used were highly enriched for NK cells, (ii) that asialo GM_1 is found at high densities on NK cells (13), and (iii) that the dilution of anti-asialo GM_1 being used did not result in labeling of the cells having low-density asialo GM_1, then the data presented below provide strong evidence that NK cells were the cells forming conjugates with *C. neoformans*.

MATERIALS AND METHODS

Mice. We used 6- to 8-week old inbred CBA/J mice purchased from the Jackson Laboratories, Bar Harbor, Maine, for this investigation.

Tumor cell target. YAC-1, a Moloney virus-induced lymphoma cell line obtained originally from Ronald Herberman, National Cancer Institute, was the cell line used routinely in the 4-h ^{51}Cr release assay to assess NK cell

* Corresponding author.

activity. YAC-1 cells were maintained in RPMI 1640 medium (GIBCO Laboratories, Grand Island, N.Y.) supplemented with 10% heat-inactivated fetal bovine serum, 100 μg of streptomycin per ml, and 100 U of penicillin per ml.

Mycotic target. *C. neoformans* isolate 184, a serotype A strain used throughout this study, has been described previously by Murphy and Cozad (20). The organisms were maintained on modified Sabouraud agar slants and were harvested from slants after 72 h of incubation at room temperature.

Preparation of the effector cells. Effector cells were prepared as previously described by Murphy and McDaniel (21). Briefly, mice were sacrificed by cervical dislocation. Spleens were pooled, and single-cell suspensions were prepared in Hanks balanced salt solution. Erythrocytes were removed by treating the preparation for 2 min with an ammonium chloride Tris-buffered solution, a treatment which did not significantly affect NK cell activity. The cells were washed twice in RPMI 1640 medium containing 5% fetal bovine serum before being passed through nylon wool columns. The NWN cell populations contained an average of 95% viable cells, with only 2% immunoglobulin-positive cells and 1% esterase-positive cells. To further enrich for NK cells, NWN cells were separated into five fractions on discontinuous Percoll gradients consisting of 70 to 30% Percoll (Pharmacia, Uppsala, Sweden) by using the method of Luini et al. (17). Each cell fraction was collected, washed twice in Hanks balanced salt solution, suspended in RPMI 1640 medium containing 10% fetal bovine serum, and used in the experiments. The cell viability of each cell fraction was determined by trypan blue dye exclusion.

Cytotoxicity assay against YAC-1 targets. The ^{51}Cr release assay described by Murphy and McDaniel (21) was used to determine the levels of NK cell reactivity in the cell fractions obtained from Percoll gradients. Briefly, YAC-1 cells were labeled for 1 h at 37°C with 100 μCi of radioactive sodium chromate (Amersham Corp., Arlington Heights, Ill.) and then were used as targets in a 4-h assay. The ratio of effector cells to target cells used throughout this study was 25:1. The percentage of ^{51}Cr released into the supernatant was calculated by using the following formula: % specific release = [(cpm experimental − cpm spontaneous)/cpm maximum] × 100. Experimental wells contained effector and target cells, spontaneous release wells contained ^{51}Cr-labeled target cells and medium, and maximum ^{51}Cr release wells contained labeled target cells mixed with 2 N HCl.

Immunofluorescent microscopy. Each Percoll cell fraction was treated separately with goat anti-Thy1 serum (21), rabbit anti-mouse immunoglobulin (21), or rabbit anti-asialo GM_1 (Wako Chemicals USA, Inc., Dallas, Tex.); this was followed by treatment with rabbit anti-goat serum for the anti-Thy1-treated cells or goat anti-rabbit serum in the case of anti-mouse immunoglobulin-treated cells or anti-asialo GM_1-treated cells. Each of the secondary antisera had been conjugated to fluorescein isothiocyanide (Cappel Laboratories, Westchester, Pa.). For controls, cells were treated with tissue culture medium in place of goat anti-Thy1 antibody, or normal rabbit serum to replace rabbit anti-mouse immunoglobulin serum or rabbit anti-asialo GM_1 antibody, and then the appropriate secondary antiserum was applied. Next, the cells were examined for the percentage of fluorescent cells after treatment with each antiserum to indicate the presence of Thy1, immunoglobulin, and asialo GM_1 surface markers. A total of 400 cells were counted in each case by using an Olympus model BH-2 fluorescent microscope. In some experiments, adherent cell fractions obtained from nylon wool columns were also stained with the same concentration of anti-asialo GM_1 and the secondary antibody as a control.

Nonspecific esterase staining. Each Percoll cell fraction was stained for nonspecific esterase by the method of Koski et al. (14) to assess macrophage-monocyte contamination.

Giemsa staining. Cytocentrifuged slide preparations of each Percoll cell fraction were stained with 3% aqueous Giemsa solution and examined by using a 100× objective on an Olympus microscope.

***C. neoformans* growth inhibition assay.** The growth inhibition assay which we used has been described previously (21). Briefly, 0.1-ml portions of each effector cell population (10^6 cells per ml) were mixed with 0.1-ml portions of *C. neoformans* target cells (2×10^4 cells per ml) in RPMI 1640 medium containing 10% fetal bovine serum and antibiotics (ratio of effector cells to target cells, 50:1) in duplicate in 96-well flat-bottom microtiter plates. Control wells contained the target cells and tissue culture medium. The plates were incubated for 18 h at 37°C in an atmosphere of 5% CO_2; then the contents of each well were serially diluted, and 1-ml portions of each final dilution were plated in duplicate onto modified Sabouraud dextrose agar plates. The number of *C. neoformans* CFU was determined after 3 days of incubation at room temperature, and the percentage of growth inhibition was calculated as follows: % of growth inhibition = [(mean CFU of control − mean CFU of experimental culture)/mean CFU of control] × 100.

Enumeration of effector cell-*C. neoformans* conjugates. Samples of effector cells obtained from different Percoll fractions on NWN cells were incubated with *C. neoformans* target cells at a ratio of effector cells to target cells of 2:1 for 18 h at 37°C in an atmosphere containing 5% CO_2. Then the samples were stained with 0.25% alcian blue in serum-free RPMI 1640 medium (pH 6.4) for light microscopy. The percentage of effector-target conjugates in each fraction was calculated after counting 400 effector cells.

Preparation of antibody-latex conjugates (immunolatex). Covalent binding of goat anti-rabbit antibodies (Cappel) to 0.24-μm latex spheres (Polysciences Inc., Warrington, Pa.) was done by using a modification of the method of Gamliel et al. (7). While being stirred at room temperature, 0.2 ml of 2.5% solid monodispersed latex spheres and 0.05 ml of diluted antiserum containing 250 μg of protein per ml were added to 4.65 ml of 0.01 M phosphate buffer (pH 9.4). The final volume was brought to 5.0 ml by adding 0.1 ml of 1% glutaraldehyde. The reaction mixture was stirred for 2 h, quenched with 0.1 M glycine, and dialyzed against phosphate buffer (pH 9.4) for 24 h at 4°C. The conjugated latex preparation was centrifuged at 17,400 × *g* for 25 min, and the pellet was suspended in phosphate-buffered saline (pH 9.4). The large aggregates were removed by centrifugation at 1,940 × *g* for 20 min before the conjugated latex spheres were used for indirect labeling of cells.

Cell labeling and specimen preparation for SEM. Effector cells obtained from Percoll fractions 1 and 2 were adjusted to a concentration of 10^7 cells per ml, and 0.1 ml of this preparation was incubated with 0.1 ml of *C. neoformans* at a concentration of 5×10^6 cells per ml (ratio of effector cells to target cells, 2:1). For controls, 0.1 ml of the *C. neoformans* target cells was incubated in the presence of an equal volume of tissue culture medium for 18 h at 37°C. After incubation, the effector cell-target cell mixtures or target cells alone were fixed in 1% glutaraldehyde in phosphate-buffered saline (pH 7.0) at room temperature. Excess glutaraldehyde was removed by incubating the cells with 0.1 M glycine in phosphate-buffered saline (pH 7.0) for 30 min, followed by

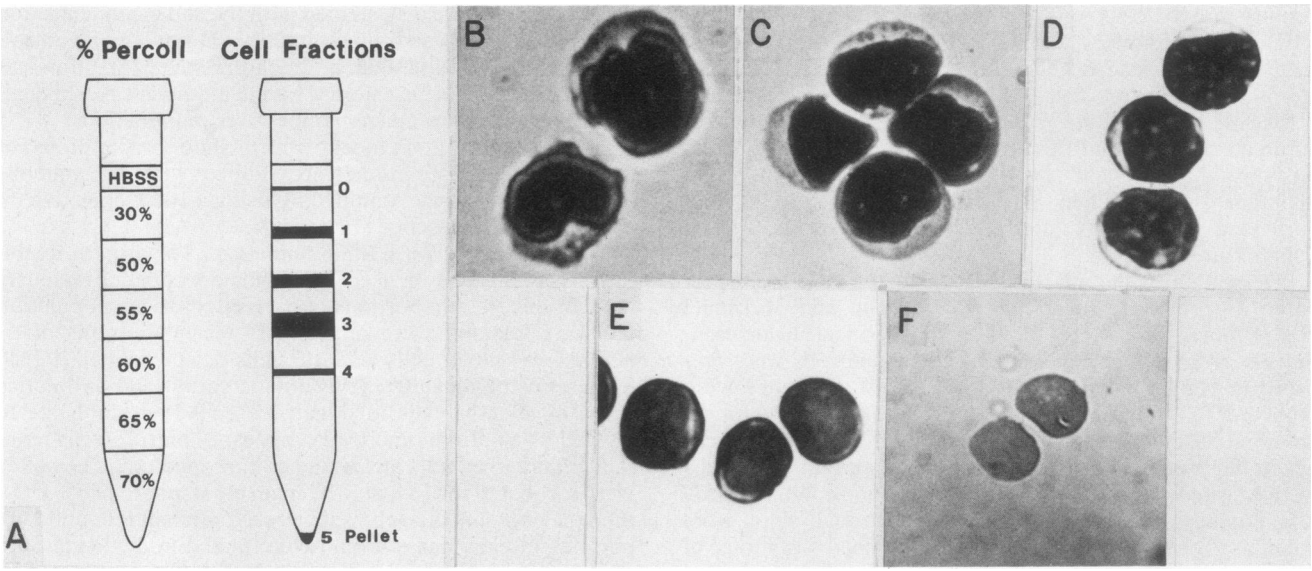

FIG. 1. (A) Fractionation of NWN splenic cells on a five-step discontinuous Percoll gradient. HBSS, Hanks balanced salt solution. (B through F) Light micrographs of Giemsa-stained cytocentrifuged cells revealing large granular and agranular lymphocytes with acentric nuclei in fractions 1 and 2 (B and C, respectively), small lymphocytes in fractions 3 and 4 (D and E, respectively), and erythrocytes in the pellet (F). ×1,300.

two washes with phosphate-buffered saline (pH 7.0) containing 0.1% bovine serum albumin. Next, the cells were treated with 0.05 ml of a 1:40 dilution of rabbit anti-asialo GM_1 or normal rabbit serum as a control for 30 min at room temperature. After two washes with phosphate-buffered saline containing 0.1% bovine serum albumin (pH 7.0) and a final wash with phosphate-buffered saline containing 0.1% bovine serum albumin (pH 9.4), the cells were treated for 30 min at room temperature with 0.1 ml of monodispersed latex spheres conjugated to goat anti-rabbit antiserum. The unbound latex spheres were removed by three washes with phosphate-buffered saline containing 0.1% bovine serum albumin (pH 9.4) and low-speed centrifugation. The labeled cells were stored in cacodylate buffer–0.1 M sucrose–1% glutaraldehyde overnight at 4°C. Then the cells were mounted on poly-L-lysine (Sigma Chemical Co., St. Louis, Mo.)-coated cover glasses (Carolina Biological Supply, Burlington, N.C.), treated with 1% osmium tetroxide at 4°C for 1 h, and washed with cacodylate buffer. The cells were dehydrated with a series of increasing concentrations of ethanol in water and subjected to critical point drying. The cover glasses were mounted on specimen stubs, and this was followed by sputter coating with gold-palladium. Samples were examined with an Etec Autoscan microscope.

RESULTS

Effect of Percoll fractionation of NWN splenic cells on *C. neoformans* growth-inhibitory activity. Effector cell-target cell conjugates can be more frequently observed if highly purified populations of NK cells are used; therefore, after obtaining the NWN splenic cell fraction, we further enriched for NK cells by fractionating the NWN cells on five-step discontinuous Percoll gradients (Fig. 1A). To establish that NK cell enrichment was being achieved by Percoll fractionation, the morphology, phenotype, and function of the splenic NWN cells and the cells from each Percoll fraction were determined. Light microscope examination of Giemsa-stained cytocentrifuged preparations of the cells (Fig. 1B through F) showed that the morphological characteristics of the cells in each fraction were consistent with the report of Luini et al. (17). Low-density cells from fractions 1 and 2 contained about 35% large granular lymphocytes plus some large agranular lymphocytes, as described by Kumagai et al. (16) and Itoh et al. (10); fractions 3 and 4 contained mainly small lymphocytes, and fraction 5 consisted of cell debris and erythrocyte ghosts. The phenotypes of the various cell fractions were determined by surface marker analysis, using immunofluorescent staining. As indicated in Table 1, approximately 40% of the cells in fractions 1 and 2 stained with anti-asialo GM_1, a marker found at a high density on NK cells (11, 13). Although asialo GM_1 has been reported to be found at low densities on macrophages and polymorphonuclear leukocytes of other species (9, 13), the dilution of anti-asialo GM_1 antibody used in this study in the indirect immunofluorescence assay did not label the adherent fraction of murine splenic cells. Fractions 1 and 2 had 21 and 17%, respectively, Thy1-positive cells, but virtually no immunoglobulin-positive cells. Finding Thy1-positive cells in fractions 1 and 2 was not unexpected since Thy1 is found on some, but not all, NK cells (18). On the other hand, fraction 3 consisted predominantly of small lymphocytes, with 54% of the cells having Thy1 antigen on them, an insignificant percentage of immunoglobulin-positive cells, and only 6% asialo GM_1-positive cells, indicating that fraction 3 was the T-cell-enriched population. Fraction 4 cells were morphologically and phenotypically similar to cells from fraction 3, with approximately 30% Thy1-positive cells. Fraction 5 cells did not have any of the markers described above. All fractions were free of significant macrophage contamination, as indicated by the 0 to 1% esterase-positive cells (Table 1). The NK functional activity of the NWN fraction and each Percoll cell fraction was determined with a 4-h ^{51}Cr release assay, using YAC-1 tumor cells; a reduced ratio of effector cells to target cells (25:1) was used due to insufficient yields from certain cell fractions (Fig. 2). The

TABLE 1. Characteristics of Percoll-fractionated NWN murine splenic cells

Percoll fraction	No. of cells recovered ($\times 10^6$)	% Input recovered	% of:				
			Large granular lymphocytes	Asialo GM_1-positive cells	Thy1-positive cells	Mouse immunoglobulin-positive cells	Nonspecific esterase-positive cells
NWN			6	9	75	2	1
1	0.7	3	41	44	21	0	0
2	4.2	21	32	36	17	0	0
3	10.7	53	5	6	54	1	1
4	1.8	9	1	3	29	0	0
5	0.3	1	0	0	2	0	0

cells from fractions 1 and 2 demonstrated approximately 77% of the total measurable YAC-1 cytotoxicity. These observations confirmed that Percoll fractions 1 and 2 were enriched for NK cells, whereas fractions 3 through 5 had low to insignificant activities against YAC-1 targets, indicating reduced numbers of NK cells.

Once we established that splenic cells with NK cell morphology, surface phenotype, and functional activity against YAC-1 targets were isolated in Percoll fractions 1 and 2, then the growth-inhibitory activities of these NK cell-rich fractions, as well as the activities of the NWN cells and the NK cell-deficient fractions (i.e., fractions 3 through 5), were tested against *C. neoformans* by using a ratio of effector cells to target cells of 50:1, which was one-tenth the ratio used in previous studies (21). Our results (Fig. 2) demonstrated that almost 80% of the total measurable growth-inhibitory activity against *C. neoformans* was present in the NK cell-rich fractions. Furthermore, the anti-*Cryptococcus* activities of fractions 1 and 2 were approximately three to four times higher than the activity of the NWN cell population when the preparations were tested in identical assays. Fraction 3 had low growth-inhibitory activity, and fractions 4 and 5 did not show significant levels of anti-*Cryptococcus* activity.

***C. neoformans*-binding cells.** Since NK cells form conjugates with their tumor cell targets before the lytic event (9, 24), the possibility of effector cell-*Cryptococcus* conjugate formation in the in vitro growth inhibition system was investigated by using light microscope examination of alcian blue-stained preparations of effector cell-*C. neoformans* mixtures. Alcian blue served as a differential stain, with cryptococcal cells staining under the conditions used and the effector cells remaining unstained. To assay the *Cryptococcus* binding capacity of the different effector cell populations, NWN cells and Percoll cell fractions 1 through 5 were used against *C. neoformans* at a ratio of effector cells to target cells of 2:1 to give sufficient numbers of conjugates to be observed microscopically. The percentage of *Cryptococcus*-binding cells (i.e., the percentage of conjugates) for each of the effector cell-target cell mixtures is shown in Fig. 2. The numbers of effector cell-*C. neoformans* conjugates were directly related to the NK activities of the effector cell populations (correlation coefficient, 0.96). For example, the NK cell-rich fractions (i.e., fractions 1 and 2) contained higher numbers of *Cryptococcus*-binding cells than did the NWN cell population from which the fractions were isolated. In contrast, the NK cell-deficient populations (fractions 3 through 5), which had low percentages of large granular lymphocytes and asialo GM_1-positive cells, had reduced numbers of conjugates compared with the percent-

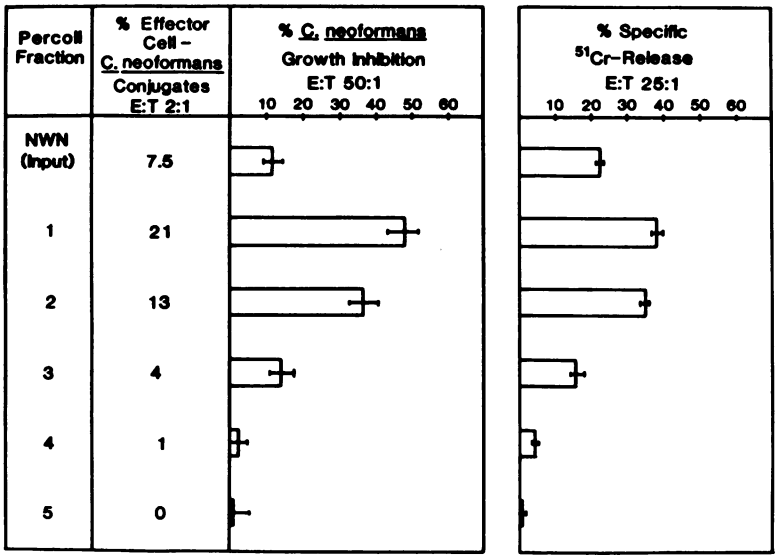

FIG. 2. Comparison of activities of cells from the five Percoll fractions in the ^{51}Cr release assay against YAC-1 target cells and in the cryptococcal growth inhibition assay. Ratios of effector cells to target cells (E:T) of 25:1 and 50:1 were used in the ^{51}Cr release and growth inhibition assays, respectively. The percentages of effector cell-*C. neoformans* conjugates were calculated as follows: percentage of conjugates = (number of effector cells bound to cryptococci/total number of effector cells) × 100.

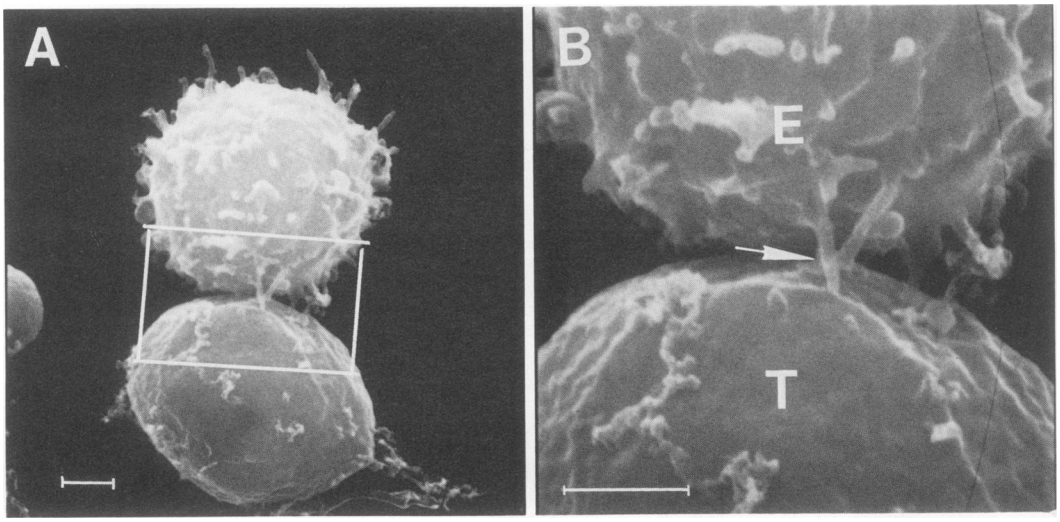

FIG. 3. (A) Scanning electron micrograph of effector cell-*C. neoformans* conjugate. (B) Higher magnification of area in box in (A). The arrow indicates appendages of the effector cell (E) extended toward the cryptococcal target cell (T). Bars = 1.0 μm.

age of conjugates in the unfractionated NWN population (Fig. 2).

SEM studies of effector cell-*C. neoformans* conjugates. To gain a higher level of resolution and obtain a more detailed view of the effector cell-target cell interactions and morphology, samples of the effector cell-*Cryptococcus* mixtures, effector cells alone, or *C. neoformans* cells alone were processed for SEM after incubation for 18 h by using identical preparation conditions for all samples. Since the level of cell recovery from fraction 1 was very low (approximately 1 to 3% of the total input [Table 1]), fractions 1 and 2 were pooled and used against *C. neoformans* for these studies.

In the scanning electron microscope, the effector cells appeared as generally homogenous cell populations in terms of size, morphology, and density of surface projections. Almost all effector cells displayed a uniform and rather dense array of microvilli and delicate ruffles on their spherical surfaces (Fig. 3 through 5). On the other hand, *C. neoformans* cells were variable, both in size and in surface morphology. Heterogeneity was expected for the cryptococ-

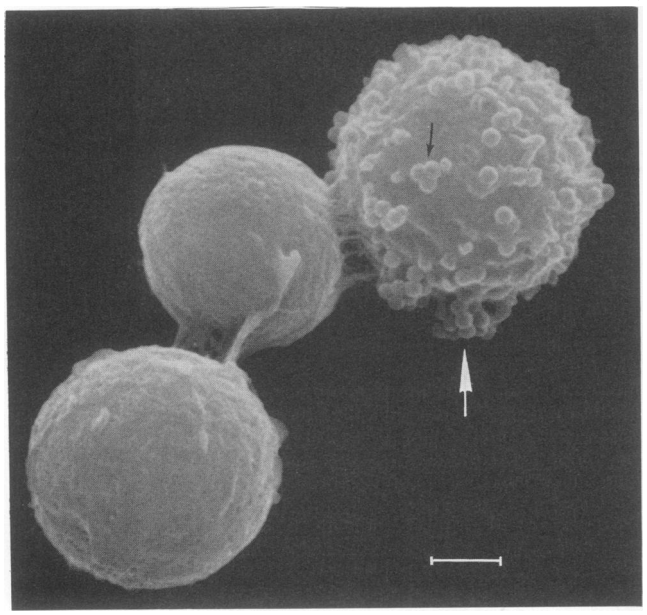

FIG. 4. Scanning electron micrograph of effector cell-*C. neoformans* conjugates stained with rabbit anti-asialo GM_1, followed by goat anti-rabbit antibody-conjugated latex spheres. The arrows indicate the latex spheres on the effector cell. Bar = 1.0 μm.

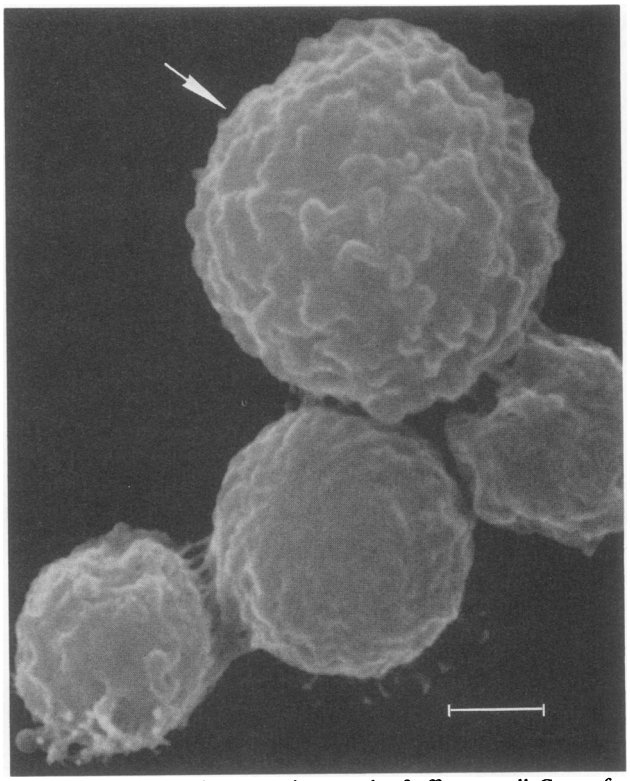

FIG. 5. Scanning electron micrograph of effector cell-*C. neoformans* conjugates treated with normal rabbit serum, followed by goat anti-rabbit antibody-conjugated latex spheres in a control experiment. The arrow indicates the effector cell. Bar = 1.0 μm.

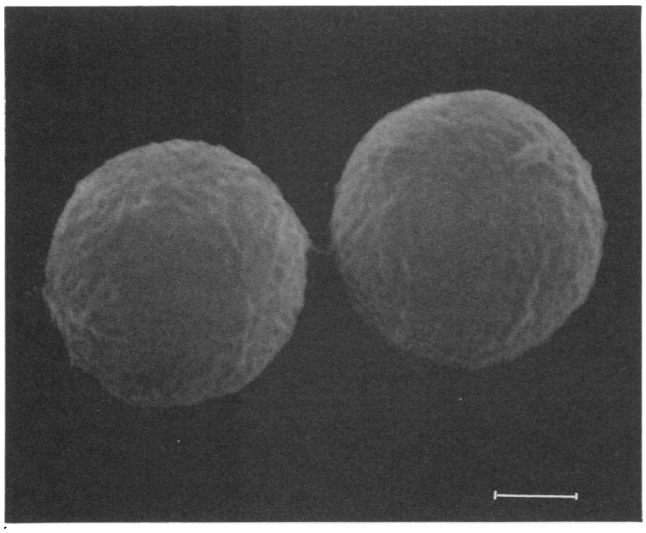

FIG. 6. Representative scanning electron micrograph of *C. neoformans* treated with anti-asialo GM_1, followed by goat anti-rabbit antibody-conjugated latex spheres. Bar = 1.0 μm.

cal cells because they vary in size from 4 to 20 μm and budding forms are frequent. The cryptococcal cells appeared to be spherical to oval shaped with relatively smooth surfaces having occasional fragments of a lacy material which was most likely capsular remnants. The capsular material was apparently partially removed from the cells during the preparation for SEM studies. In the SEM images of effector cell-*C. neoformans* conjugates, the effector cells usually had a broad contact area with the cryptococcal targets, and in virtually all cases we observed appendages connecting the effector cells to the targets (Fig. 3 through 5) (19; Murphy, Curr. Top. Med. Mycol., in press). Once we established visualization of the effector cell-*C. neoformans* conjugates with SEM, we wanted to demonstrate that the effector cells attached to *C. neoformans* cells had NK cell-associated surface markers by using an indirect immunoelectron microscopy method. This method included treating the cells with rabbit anti-asialo GM_1 as the primary antibody, followed by goat anti-rabbit antibody conjugated to latex spheres as the secondary antibody system. In all of the cases observed the lymphocytes attached to cryptococci were labeled with immunolatex spheres (Fig. 4). In control experiments in which the cells were treated first with normal rabbit serum in place of rabbit anti-asialo GM_1 and then with goat anti-rabbit antibody-conjugated latex spheres, the effector cells attached to *C. neoformans* targets remained free of latex spheres (Fig. 5), indicating that the effector cells had been specifically labeled by this method. *C. neoformans* cells associated neither with the anti-asialo GM_1 nor with normal rabbit serum; therefore, they remained free of latex spheres in both cases (Fig. 6). In the SEM preparations of the effector-*C. neoformans* cell mixtures, both budding and nonbudding yeast cells were observed in direct contact with the effector cells. Although *C. neoformans* and effector cells could be easily differentiated by electron microscopy, we selected only the conjugates which had budding *C. neoformans* cells, so that it would be absolutely clear to those viewing only a minimal number of photomicrographs which cells were the effectors and which cells were the targets.

Phagocytosis of *C. neoformans* by the effector cells or the tendency toward phagocytosis of the crytococcal targets was not observed during the SEM studies of the effector cell-*C. neoformans* conjugates (Fig. 3 through 5) (19; Murphy, in press). This might be expected since the effector cells collected from the low-density fractions (i.e., fractions 1 and 2) were virtually free of phagocytic cells, such as macrophages and polymorphonuclear leukocytes (Fig. 1 and Table 1). These observations indicate that growth inhibition of cryptococci was by a nonphagocytic mechanism, and they strengthen the view that NK cells, not phagocytic cells, were responsible for *C. neoformans* growth inhibition in our system.

DISCUSSION

In this paper we offer direct evidence that the effector cells collected from NK cell-rich Percoll gradient cell fractions 1 and 2 were capable of binding to *C. neoformans* targets and forming effector cell-target cell conjugates similar to those reported by other workers in NK cell-tumor systems (6, 24, 25). Furthermore, the number of effector cell-*C. neoformans* conjugates were directly proportional to the levels of NK cell activity of the effector cell populations used.

In previous studies (21), it was shown that the cytotoxicities of YAC-1 target cells induced by various murine splenic effector cell populations directly correlated with the growth-inhibitory activities of the effector cells against *C. neoformans*. When adherent cells were removed from the effector cells by nylon wool passage, both NK cell activity against YAC-1 target cells and growth-inhibitory activity against cryptococci increased. In the present study, when we further enriched for NK cells by fractionation of the NWN splenic cells through five-step discontinuous Percoll gradients, the NK cell activity against YAC-1 target cells, as well as the growth-inhibitory activity against cryptococci, was enhanced two- to fivefold in NK cell-rich Percoll fractions 1 and 2. Furthermore, with NK cell-rich effector cell populations, an effector cells to target cells ratio one-tenth that used in previous investigations could be used for the assay, and a substantial level of *Cryptococcus* growth inhibition was achieved with this significantly lower ratio. Percoll fractions 1 and 2, which were virtually free of macrophages, as shown by their esterase-negative characteristics, inhibited the growth of cryptococci considerably better than NWN cell preparations, which contained 1% macrophages, when used in identical assays. These results eliminate the possibility that contaminating macrophages were involved in inhibiting *C. neoformans* growth. Furthermore, phagocytosis, which is generally a prerequisite for macrophage killing, was not observed at any time when Percoll fraction 1 and 2 effector cells were associated with cryptococci. The effector cells had a broad contact area with *C. neoformans* targets; however, they did not have pseudopodial projections, nor did they show any tendencies to engulf the organisms (Fig. 3A and B). Instead, the appendages of the effector cells extended toward the target cells and made perpendicular contact with the cryptococcal cell surfaces (Fig. 3B). Similar appendages have been observed in scanning electron micrographs by us (unpublished data) as well as by other workers (2) between NK cells and target cells which are recognized by NK cells (i.e., YAC-1 and K562 cells). These observations strengthened the concept that *C. neoformans* growth inhibition was not the result of a phagocytic mechanism. The inhibitory effects on the cryptococcal targets were exerted, most likely, after direct contact was established between the effector cells and the target cells, since conjugates could be detected earlier than growth inhibition (unpublished data). It is possible that soluble inhibitory substances (factors) are

secreted by the effector cells into the microenvironment between the two cells or into the medium, as has been suggested in the NK cell-tumor cytolytic models (2, 5, 28). The inhibitory factors could be associated with the large granules seen in NK cells (8, 23).

In the SEM studies of the effector cell-*C. neoformans* conjugates, effector cells in direct contact with cryptococci specifically bound anti-asialo GM_1 antibody. The rabbit anti-asialo GM_1 antibody used in the immunoscanning electron microscopy experiments has been shown to be relatively specific for NK cells in many laboratories (11–13). In addition, we have previously demonstrated that treatment of NWN cells with anti-asialo GM_1 and complement significantly reduced both NK cell activity against YAC-1 target cells and growth-inhibitory ability against *C. neoformans* (21). Although asialo GM_1 has been reported to be found at low densities on macrophages and polymorphonuclear leukocytes of other species (9, 13), the dilution of anti-asialo GM_1 antibody used in this study, in an indirect assay, did not label the adherent fraction of murine splenic cells. Therefore, we concluded that the effector cells in direct association with *C. neoformans* were NK cells.

Observations made during the SEM studies suggested that the NK cells were likely to recognize some structures on the cryptococcal cell wall other than the capsule. This was proposed because cryptococci or effector cell-*C. neoformans* mixtures which were subjected to additional washing steps during the immunolabeling procedures had less lacy capsular material clinging to the cryptococcal surfaces than did the cells which were prepared for studies not involving immunolabeling. Other investigators have also reported the destruction of capsular material during routine electron microscope specimen preparation (15). Despite the loss of the capsular material, effector cell appendages were firmly attached to the cryptococci and did not dissociate during immunolabeling preparation for electron microscopy. These appendages were in association with the smooth surface of the organism rather than with the lacy capsular material (Fig. 3B). Other data from our laboratory also support the idea that the effector cells do not associate with the cryptococcal capsule. For example, growth inhibition of *C. neoformans* by the effector cells was independent of the size or serotype of the cryptococcal capsules (21). Furthermore, in preliminary experiments, when *C. neoformans* culture filtrates which contained capsular polysaccharide (22) were added to effector cell-*C. neoformans* mixtures at the initiation of the growth inhibition assay, cryptococcal growth inhibition was not affected (Murphy, unpublished data) suggesting that capsular components could not block binding of the effector cells to the cryptococci. Further work must be done to establish the nature of the effector cell attachment site on the *C. neoformans* target cells.

Our data establish that the effector cells against *C. neoformans* fit the characteristics defined for NK cells. The effector cells are NWN, nonphagocytic, low density, large granular lymphocytes, immunoglobulin negative, and free of Thy1 antigen or have low-density Thy1 antigen, and they bear asialo GM_1. In this study, we demonstrated that NK cells make direct contact with cryptococcal targets without phagocytosis, indicating that the growth inhibition of the cryptococci is an extracellular event. Furthermore, we showed that the percentage of the conjugates was proportional to the level of cryptococcal growth inhibition, suggesting that association of effector cells with the cryptococcal target cells may be essential to inhibition of the growth. Further studies are being directed toward elucidating the entire mechanism of *C. neoformans* growth inhibition by NK cells.

ACKNOWLEDGMENTS

This study was supported by Public Health Service grant AI-18895 from the National Institute of Allergy and Infectious Diseases.

We thank workers at Samuel Roberts Noble Laboratory of Electron Microscopy and especially William Chissoe for taking the scanning electron micrographs.

LITERATURE CITED

1. **Bulmer, G. S., and J. R. Tacker.** 1975. Phagocytosis of *Cryptococcus neoformans* by alveolar macrophages. Infect. Immun. **11**:73–79.
2. **Carpen, O., I. Virtanen, and E. Saksela.** 1982. Ultrastructure of human natural killer cells: nature of the cytolytic contacts in relation to cellular secretion. J. Immunol. **128**:2691–2697.
3. **Diamond, R. D., and J. E. Bennett.** 1973. Disseminated cryptococcosis in man: decreased lymphocyte transformation in response to *Cryptococcus neoformans*. J. Infect. Dis. **127**:694–697.
4. **Diamond, R. D., R. K. Root, and J. E. Bennett.** 1972. Factors influencing killing of *Cryptococcus neoformans* by human leukocytes in vitro. J. Infect. Dis. **125**:367–376.
5. **Farram, E., and S. R. Targan.** 1983. Identification of human natural killer soluble cytotoxic factor(s) (NKCF) derived from NK-enriched lymphocyte populations: specificity of generation and killing. J. Immunol. **130**:1252–1256.
6. **Frey, T., H. R. Petty, and H. M. McConnell.** 1982. Electron microscopic study of natural killer cell-tumor cell conjugates. Proc. Natl. Acad. Sci. U.S.A. **79**:5317–5321.
7. **Gamliel, H., R. Leizerowitz, D. Gurfel, and A. Polliack.** 1981. Scanning immuno-electron microscopy of human leukemia and lymphoma cells: a comparative study of techniques using immunolatex spheres as markers. J. Microsc. (Oxford) **123**:189–199.
8. **Henkart, P. A., P. J. Millard, C. W. Reynolds, and M. P. Henkart.** 1984. Cytolytic activity of purified cytoplasmic granules from cytotoxic rat large granular lymphocyte tumors. J. Exp. Med. **160**:75–93.
9. **Herberman, R. B., and J. R. Ortaldo.** 1981. Natural killer cells: their role in defenses against disease. Science **214**:24–30.
10. **Itoh, K., R. Suzuki, Y. Umezu, K. Hanaumi, and K. Kumagai.** 1982. Studies of murine large granular lymphocytes. II. Tissue, strain, and age distributions of LGL and LAL. J. Immunol. **129**:395–400.
11. **Kasai, M., M. Iwamori, Y. Nagai, K. Okumura, and T. Tada.** 1980. A glycolipid on the surface of mouse natural killer cells. Eur. J. Immunol. **10**:175–180.
12. **Kawase, I., D. L. Urdal, C. G. Brooks, and C. S. Henney.** 1982. Selective depletion of NK cell activity in vivo and its effect on the growth of NK-sensitive and NK-resistant tumor cell variants. Int. J. Cancer **29**:567–574.
13. **Keller, R., T. Bächi, and K. Okumura.** 1983. Discrimination between macrophage and NK-type tumoricidal activities via anti-asialo GM1 antibody. Exp. Cell Biol. **51**:158–164.
14. **Koski, I. R., D. G. Poplack, and R. M. Blaese.** 1976. A nonspecific esterase stain for the identification of monocytes and macrophages, p. 359–362. *In* B. R. Bloom and J. R. David (ed.), In vitro methods in cell-mediated and tumor immunity. Academic Press, Inc., New York.
15. **Kozel, T. R., B. Highison, and C. J. Stratton.** 1984. Localization on encapsulated *Cryptococcus neoformans* of serum components opsonic for phagocytosis by macrophages and neutrophils. Infect. Immun. **43**:574–579.
16. **Kumagai, K., K. Itoh, R. Suzuki, S. Hinuma, and F. Saitoh.** 1982. Studies of murine large granular lymphocytes. I. Identification as effector cells in NK and K cytotoxicities. J. Immunol. **129**:388–394.
17. **Luini, W., D. Boraschi, S. Alberti, A. Aleotti, and A. Tagliabue.** 1981. Morphological characterization of a cell population responsible for natural killer activity. Immunology **43**:663–668.

18. **Mattes, M. J., S. O. Sharrow, R. B. Herberman, and H. T. Holden.** 1979. Identification and separation of Thy-1 positive mouse spleen cells active in natural cytotoxicity and antibody-dependent cell-mediated cytotoxicity. J. Immunol. **123**:2851–2860.
19. **Murphy, J. W.** 1984. Natural killer cells against a mycotic pathogen, p. 328–332. *In* L. Leive and D. Schlessinger (ed.), Microbiology—1984. American Society for Microbiology, Washington, D.C.
20. **Murphy, J. W., and G. C. Cozad.** 1972. Immunological unresponsiveness induced by cryptococcal capsular polysaccharide assayed by the hemolytic plaque technique. Infect. Immun. **5**:896–901.
21. **Murphy, J. W., and D. O. McDaniel.** 1982. In vitro reactivity of natural killer (NK) cells against *Cryptococcus neoformans*. J. Immunol. **128**:1577–1583.
22. **Murphy, J. W., and N. Pahlavan.** 1979. Cryptococcal culture filtrate antigen for detection of delayed-type hypersensitivity in cryptococcosis. Infect. Immun. **25**:284–292.
23. **Podack, E. R.** 1985. The molecular mechanism of lymphocyte-mediated tumor cell lysis. Immun. Today **6**:21–27.
24. **Roder, J. C., R. Kiessling, P. Biberfeld, and B. Andersson.** 1978. Target-effector interaction in the natural killer (NK) cell system. II. The isolation of NK cells and studies on the mechanism of killing. J. Immunol. **121**:2509–2517.
25. **Rubin, P., H. F. Pross, and J. C. Roder.** 1982. Studies of human natural killer cells. II. Analysis at the single cell level. J. Immunol. **128**:2553–2558.
26. **Schimpff, S. C., and J. E. Bennett.** 1975. Abnormalities in cell-mediated immunity in patients with *Cryptococcus neoformans* infection. J. Allergy Clin. Immunol. **55**:430–441.
27. **Swenson, F. J., and T. R. Kozel.** 1978. Phagocytosis of *Cryptococcus neoformans* by normal and thioglycolate-activated macrophages. Infect. Immun. **21**:714–720.
28. **Wright, S. C., M. L. Weitzen, R. Kahle, G. A. Granger, and B. Bonavida.** 1983. Studies on the mechanism of natural killer cytotoxicity. II. Coculture of human PBL with NK-sensitive or resistant cell lines stimulates release of natural killer cytotoxic factors (NKCF) selectively cytotoxic to NK-sensitive targets. J. Immunol. **130**:2479–2483.