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In Vitro Effects of Natural Killer Cells Against *Paracoccidioides brasiliensis* Yeast Phase

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Recently, data have been reported suggesting natural killer (NK) cells may function in natural resistance against a fungus, *Cryptococcus neoformans*. The primary objective of this study was to examine the reactivity of murine splenic cells against another fungus, *Paracoccidioides brasiliensis*. Levels of NK activity in effector cell pools were varied by: (i) removing nylon wool-adherent cells, (ii) fractionating splenic cells on Percoll discontinuous gradients, (iii) using old and young effector cell donor mice, (iv) using donors from different strains, and (v) pretreating donors with NK-augmenting and -depressing agents. The various effector cell pools were simultaneously used in the 4-h ^{51}Cr release assay with YAC-1 targets to determine the NK reactivity and in the in vitro growth inhibition assay against *P. brasiliensis* yeast phase targets. In each case, the level of NK reactivity correlated with the ability of the effector cells to inhibit the in vitro growth of *P. brasiliensis*. NK activity and *P. brasiliensis* growth-inhibiting ability could be augmented by fractionation of splenic cells through nylon wool or Percoll gradients. The effector cells responsible for the NK activity and *P. brasiliensis* growth inhibition were characterized as being nylon wool nonadherent, being found in the low-density fractions from Percoll discontinuous gradients, and having no detectable Thy-1 antigen or immunoglobulin but having asialo GM1 on their surface. These data support the contention that NK or NK-like cells are responsible for limiting the in vitro growth of *P. brasiliensis*.

Paracoccidioides brasiliensis, a dimorphic fungus with an exogenous habitat (2, 40), causes a granulomatous disease called paracoccidioidomycosis (South American blastomycosis) in susceptible individuals. This organism is found in nature in the mycelial phase, and, generally, spore formation is infrequent (44, 46). The disease is thought to be acquired by inhalation of the organism and is manifested in diverse clinical forms, which range from asymptomatic pulmonary lesions to generalized infections with the invasion of many organs (8, 29). The tissue form of *P. brasiliensis* is a multiple-budding yeast cell. Paracoccidioidomycosis is limited to humid tropical and subtropical areas of continental Latin America and predominantly affects rural males of fully productive ages (5, 12, 28). As in other deep mycoses, many people are exposed to the organism, but only a small number develop the clinical disease (1, 12, 49), suggesting the presence of innate and acquired resistance mechanisms in the hosts. Acquired immunity, both humoral and cell-mediated, has been the most studied host defense mechanism against this disease (7, 33, 39, 47, 48). On the other hand, the effectiveness of natural, cell-mediated mechanisms is still virtually undefined (10, 11, 50).

The demonstration by Murphy and McDaniel (37) that natural killer (NK) or NK-like cells can inhibit the growth of the yeast-like organism *Cryptococcus neoformans* stimulated us to determine whether naturally occurring nonphagocytic cells had the potential to play a defensive role against *P. brasiliensis*.

NK cells were first described in the mid 1970's and have been shown to be cytotoxic to tumor (reviewed in references 15, 16, 18, and 24) and virus-infected cells (4, 52), suggesting that they may be important in primary host defense. More recently, natural cytotoxic cells have been implicated as effector cells against mycotic (3, 36, 37), parasitic (14), and bacterial agents (41). NK cells have been found in lymphoid tissues, except thymus tissue, of normal nonimmunized individuals of many different species (17). Murine NK cell activity has been shown to be absent at birth, being detectable between 3 to 5 weeks, reaching a peak between 6 to 8 weeks, then decreasing with age (18, 24, 55). Different strains of mice (18, 24) have varying levels of NK activity, and the activity has been modulated by such agents as *Corynebacterium parvum* (13, 53). NK cells have been characterized as being nonadherent (17), nonphagocytic (17), large granular lymphocytes (26, 30, 51, 54), without detectable amounts of surface immunoglobulins or significant concentrations of Thy-1 antigen (17). Surface markers reportedly on NK cells are asialo GM1 (a glycosphingolipid) (22), NK-1 (6, 9, 25), and Qa4 and Qa5 antigens (9).

The primary objective of this study was to examine the ability of murine splenic cells with NK cell characteristics to inhibit the growth of *P. brasiliensis*. By varying the levels of NK activity in effector cell pools, we have shown that NK activity, as measured by the in vitro 4-h ^{51}Cr release assay against YAC-1 targets, correlated with the inhibition of in vitro growth of *P. brasiliensis* yeast cells. Furthermore, the effector cells responsible for the *P. brasiliensis* growth-inhibiting activity had characteristics of murine NK cells. They were nylon wool nonadherent (NWN), were found in the low-density cell fractions from Percoll discontinuous gradients, and had no detectable Thy-1 antigen or immunoglobulin on their surfaces but did have asialo GM1.

MATERIALS AND METHODS

Mice. CBA/N and A.TH mice were bred in the University of Oklahoma animal facilities. The original breeding stock of CBA/N mice was obtained from the National Institutes of Health, and the breeding stock of A.TH mice was provided by Donald Shreffler, Washington University, St. Louis, Mo.

Target cells. YAC-1 cells, a Moloney virus-induced line,

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obtained from R. B. Herberman, National Cancer Institute, were used to assess the NK activity. The cells were maintained in our laboratory in RPMI 1640 medium supplemented with 10% fetal calf serum (GIBCO Laboratories, Grand Island, N.Y.), 100 U of penicillin per ml, and 100 μg of streptomycin per ml (complete RPMI 1640). *P. brasiliensis* isolates LA, C81, B339, GA, GI, and CA, used as targets in the growth inhibition assays, were obtained from A. Restrepo, Corp. of Investigative Biology, Medellin, Colombia. The yeast phase of *P. brasiliensis* was maintained on modified McVeigh-Morton agar slants (45). Fresh slants were inoculated and incubated at 37°C for 72 h before the organisms were harvested, washed, and adjusted to the desired concentration in complete RPMI 1640.

Preparation of effector cells. Single-cell suspensions were prepared by pressing spleens through a 200-mesh stainless screen into sterile Hanks balanced salt solution. Erythrocytes were removed from the cell pools by lysing the erythrocytes with Tris–ammonium chloride buffer (pH 7.2) (32), followed by two washings with Hanks balanced salt solution. The cells were suspended in cold RPMI 1640 culture medium plus 5% fetal calf serum. NK-enriched cell populations were obtained by collecting nonadherent splenic cells from nylon wool columns (NWC) as described previously (21). Approximately 3×10^8 splenic cells were put on a 1.4-g NWC, and 25 to 30% of the cells were recovered in the nonadherent fractions. Effector cells were adjusted to 10^7 cells per ml in complete RPMI 1640.

In vitro cytolytic assay. YAC-1 target cells were suspended in serum-free RPMI 1640 at a concentration of 10^7 cells per ml. The cells were labeled with 100 μCi of radioactive sodium chromate (New England Nuclear Corp., Boston, Mass.) for 1 h at 37°C in 5% CO_2. After three washings to remove the excess unbound ^{51}Cr, the target cell suspensions were adjusted to 2×10^5 cells per ml in complete RPMI. The effector (10^7 cells per ml) and target cell suspensions were added to quadruplicate wells of U-bottomed microtiter plates (Linbro Scientific Co., Hamden, Conn.) in 0.1-ml volumes. The plates were incubated at 37°C in 5% CO_2 for 4 h. After centrifugation, 0.1 ml of supernatant was removed, transferred to disposable glass tubes (12 by 75 mm), and counted in a gamma counter (Beckman Instruments, Inc., Fullerton, Calif.) for 10 min. Spontaneous release was determined by calculating the mean counts in supernatants from quadruplicate wells which contained 0.1 ml of the target cell suspension and 0.1 ml of complete RPMI 1640. The maximun release was determined by calculating the mean counts of supernatants from wells which contained 0.1 ml of target cells lysed with 0.1 ml of 2 N HCl. Cytotoxicity of YAC-1 targets was expressed by using the following formula: % lysis = (experimental release − spontaneous release/maximum release) × 100.

In vitro growth inhibition assay. *P. brasiliensis* yeast cells to be used as target cells were harvested from agar slants, washed three times, and adjusted to 2×10^4 cells per ml in complete RPMI 1640. Quadruplicate wells in flat-bottomed microtiter plates (Linbro) were prepared by adding 0.1 ml of target and effector cell suspensions to each well. Controls containing 0.1 ml of the target cell suspension and 0.1 ml of complete RPMI 1640 were also run in quadruplicate. To each well, 0.05 ml of complete RPMI 1640 was added to give a final volume of 0.25 ml per well. The plates were incubated at 37°C for 18 h in 5% CO_2 in a humidified atmosphere. After incubation, the contents of the wells were mixed by shaking the plates for 45 s on a Micro-Shaker II (Dynatech Laboratories, Inc., Alexandria, Va.). Samples were removed from each well, diluted, and plated in duplicate on modified Sabouraud glucose agar medium. Colonies were counted 2 weeks after the suspensions were plated, and the percent growth inhibition was determined by the following formula: % inhibition of growth = $C - T/C \times 100$, where C is the mean number of colonies on plates from control wells and T is the mean number of colonies on plates from test wells.

Procedures for augmenting NK activity. Formalin-killed

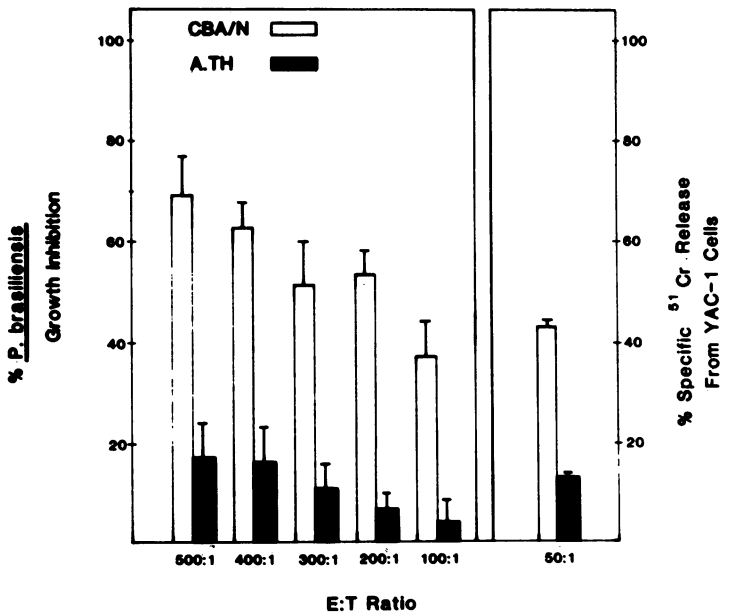

FIG. 1. Reactivity of NWN splenic cells from 7-week-old CBA/N or A.TH mice in the 4-h ^{51}Cr release assay against YAC-1 targets and in the 18-h growth inhibition assay against *P. brasiliensis* targets. Five different E:T ratios were used in the growth inhibition assay. Bars indicate the mean and SEM based in at least four experiments.

Corynebacterium parvum was obtained from Richard L. Tuttle, Burroughs Wellcome Co., and 0.7 mg of *C. parvum* per mouse was injected intravenously into 24-week-old CBA/N mice at 3 or 7 days before spleen cells were collected for use in the assays for NK cell activity or *P. brasiliensis* growth inhibition.

Antiserum treatment of the cells. Anti-Thy-1 serum has been described previously (38). Anti-mouse immunoglobulin was supplied and characterized (34, 35, 42) by John Moorhead, University of Colorado Health Science Center. Anti-asialo GM1 (21) was obtained from Wako Pure Chemical Industries, Dallas, Tex. Agarose-absorbed guinea pig serum (31) at a 1/32 dilution served as the complement. In assays to determine surface characteristics of effector cells, NWN splenic cell pools were incubated with the relevant antiserum for 1 h at 4°C, followed by two washings with Hanks balanced salt solution. After the final wash, complement was added to the cell pellets, then the contents of the tubes were mixed and incubated for 10 min at 4°C, followed by incubation at 35 min at 37°C. The cells were washed and suspended in the appropriate volume of complete RPMI 1640 and were used immediately in the ^{51}Cr release assay against YAC-1 targets and in the growth inhibition assay against *P. brasiliensis* targets.

Enrichment of effector cells by using density gradients. Discontinuous density gradients were prepared with Percoll (Pharmacia Fine Chemicals, Uppsala, Sweden) by the method of Luini et al. (30). NWN cells (2 ml), at a concentration of 2×10^6 cells per ml were added to the top of the gradient, and the tubes were centrifuged at 4°C. After centrifugation at $300 \times g$ for 30 min, the top two low-density fractions, designated fractions 1 and 2, were collected. Cells in fractions 1 and 2 had the highest percentages of large granular lymphocytes (30; J. W. Murphy, *in* M. R. McGinnis [ed.], *Current Topics in Medical Mycology*, in press) and had characteristics of NK cells (Murphy, in press). These cells were used as effectors in the 4-h ^{51}Cr release assay with YAC-1 targets to define the NK activity and in the growth inhibition assay against *P. brasiliensis* yeast-phase targets.

Statistical analysis. Means, standard errors of the means (SEM), and unpaired *t* tests programmed on an Apple II plus computer were used in the analyses of data.

RESULTS

Effects of varying E:T ratios on inhibition of *P. brasiliensis* growth. The first series of experiments were done to determine whether NK activity of the various effector cell populations correlated with their ability to inhibit the growth of *P. brasiliensis*. To evaluate the levels of NK reactivity for each of these murine cell suspensions, a standard assay for determining murine NK cell activity (23) was used, the 4-h ^{51}Cr release assay with YAC-1 cells as targets. Initially, different effector-target (E:T) ratios ranging from 100:1 to 500:1 were used in the 18-h growth inhibition assay against *P. brasiliensis*. Comparisons were made between the NK activity and the *P. brasiliensis* growth-inhibiting activity of spleen cells from 7-week-old CBA/N and A.TH mice, using various E:T ratios in the growth inhibition assay (Fig. 1). CBA/N splenic cell pools had higher NK reactivity and inhibited the growth of *P. brasiliensis* significantly better ($P < 0.005$) than did A.TH splenic cells. A 500:1 E:T ratio exhibited the highest percent inhibition of *P. brasiliensis* growth; therefore, this ratio was routinely used in all subsequent experiments.

Susceptibility of various isolates of *P. brasiliensis* to inhibition by effector cells. To demonstrate that *P. brasiliensis*

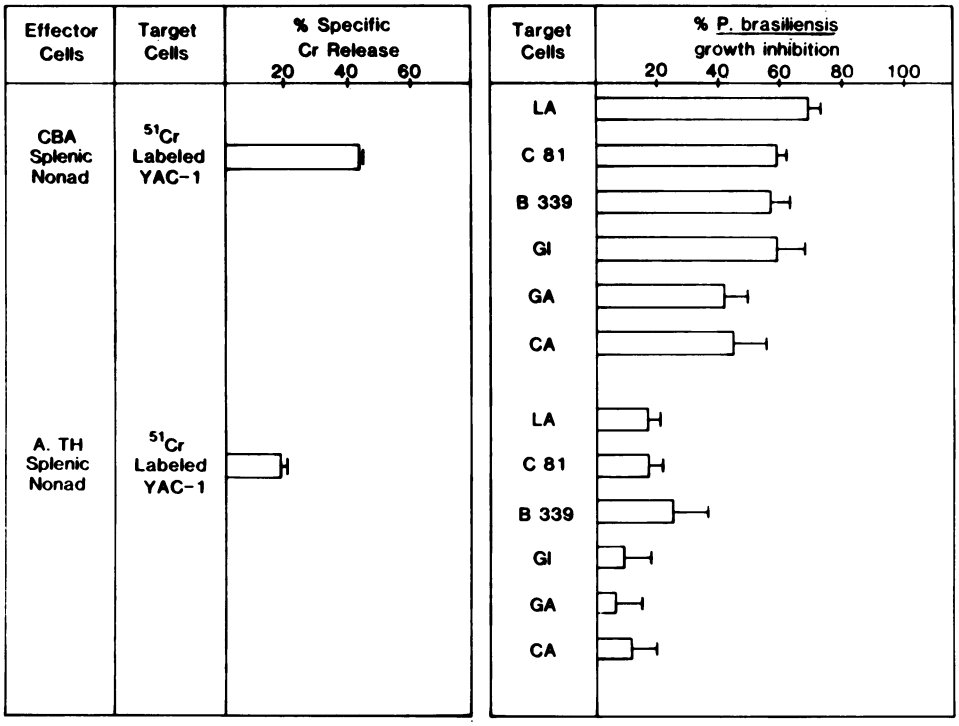

FIG. 2. Reactivity of NWN splenic cells from 7-week-old CBA/N or A.TH mice against ^{51}Cr-labeled YAC-1 targets or *P. brasiliensis* yeast phase cells. Six different isolates of *P. brasiliensis* were used in the growth inhibition assay. Bars indicate mean and SEM based on four experiments.

TABLE 1. Comparison of cytolytic and growth-inhibiting activities of unseparated and NWN cells from 7-week-old CBA/N and A.TH mice when used against YAC-1 targets or *P. brasiliensis* yeast phase cells. Mean and SEM based on four experiments

Splenic effector cells	% Inhibition of *P. brasiliensis* growth (500:1 E:T ratio)		% Specific cytolysis of YAC-1 (50:1 E:T ratio)	
	CBA/N	A.TH	CBA/N	A.TH
Unseparated	29 ± 3	7 ± 8	11 ± 2	2 ± 3
NWN	57 ± 3	19 ± 3	44 ± 3	9 ± 3
Comparison (*P*)	<0.0005	<0.05	<0.0005	<0.1

isolate LA, which was used in the majority of the studies presented here, was not the only isolate susceptible to NK activity, five other isolates were used in the growth inhibition assay. The results are shown in Fig. 2. Regardless of the *P. brasiliensis* isolate used as the target, CBA/N splenic cells with high NK activity were able to inhibit growth better than were A.TH spleen cells, which had low NK activity.

Enrichment of NK and *P. brasiliensis* growth-inhibiting activity. A subsequent series of experiments was devoted to enrich for NK cells by passing the effector cells over NWC. Table 1 shows the results when the bulk of the macrophages and B cells were removed from effector cell pools. The activities of NWN fractions were higher in both assays than were the activities of the unseparated splenic cells. Augmentation of *P. brasiliensis* growth-inhibiting activity was demonstrated even when NWN splenic cells were used at an 100:1 E:T ratio (Table 2). Therefore, NWN splenic cells were routinely used. Further enrichment of NK activity was pursued by fractionating NWN splenic cells from 7-week-old mice by using discontinuous Percoll density gradients. This method has been shown to augment the NK activity of human (54) and mouse (30) mononuclear cells. Cells from Percoll fractions 1 and 2 were pooled, owing to low yields, and were used in the ^{51}Cr release and growth inhibition assays. The activities of Percoll fractions 1 and 2 cells were substantially enriched against YAC-1 and *P. brasiliensis* targets when compared with activities of NWN cells (Table 2). When NWN splenic cells were used at a 500:1 effector:*P. brasiliensis* ratio (Table 1) in the growth inhibition assay, ca. 60% inhibition was achieved; however, by enriching for large granular lymphocytes on Percoll gradients (Table 2), 67% inhibition of *P. brasiliensis* growth was achieved with an E:T ratio of 100:1. To compare NK activity with the ability to inhibit the growth of *P. brasiliensis*, correlation coefficients were calculated by using the combined data from four experiments. When the two activities were compared, the correlation coefficient with unseparated splenic cells was 0.82; with NWN splenic cells, it was 0.91; and with cells from Percoll fractions 1 and 2, it was 0.98.

TABLE 2. Comparison of activities of unseparated, NWN, and Percoll fractions 1 and 2 splenic cells from 7-week-old CBA/N mice in the growth inhibition assay against *P. brasiliensis* targets and in the ^{51}Cr release against YAC-1 targets. Mean and SEM based on four experiments

Effector	% Inhibition of *P. brasiliensis* growth (100:1 E:T ratio)	% Specific cytolysis of YAC-1 (50:1 E:T ratio)
Unseparated splenic cells	35 ± 1	19 ± 1
NWN cells	52 ± 6	38 ± 1
Percoll	67 ± 5	55 ± 2

TABLE 3. Comparison of the reactivities of NWN splenic cells from 7-week-old and 24-week-old CBA/N mice in the growth inhibition assay against *P. brasiliensis* targets and in the 4-h ^{51}Cr release assays against the YAC-1 targets. Mean and SEM based on three experiments

Age (wk)	% Inhibition of *P. brasiliensis* growth	% Specific cytolysis of YAC-1
7	60 ± 7	34 ± 3
24	35 ± 10	16 ± 1
Comparison (*P*)	<0.025	<0.0005

Effects of varying NK activity on *P. brasiliensis* growth inhibition. Because NK activity varies with the age of the mice, experiments were done to examine the effects of age on the *P. brasilienses* growth inhibition assay. Effector cells from old (24 weeks) and young (7 weeks) CBA/N mice were used in the two assays. Effector cells from 7-week-old CBA/N mice were significantly more effective than those from 24-week-old mice against both types of targets (Table 3). When the data from the two age groups were compared by the *t* test, the *P* values were 0.025 for the *P. brasiliensis* growth inhibition and 0.0005 for the ^{51}Cr release assay. To further examine whether levels of growth inhibition varied in parallel with NK activity, an NK-enhancing agent, *C. parvum* was used in the next series of experiments. At 3 days after *C. parvum* treatment, murine splenic cells have been shown to have augmented NK activity; however, by 7 days posttreatment, the NK activity was slightly depressed (13, 53). Figure 3 shows the activities of splenic cells from CBA/N mice treated with *C. parvum* either 3 or 7 days before the cells were harvested for the assays. Both NK and growth inhibitory activities were enhanced significantly 3 days after *C. parvum* injection; however, by 7 days, the activities were reduced to or below the level for cells from untreated animals.

Characterization of effector cells. To determine whether cells with the same antigenic characteristics as NK cells were the effectors in the *P. brasiliensis* growth inhibition system, the NWN splenic cells were treated with anti-Thy-1, anti-mouse immunoglobulin, or anti-asialo GM1 antisera and complement before being used as effector cells in the two assays (Fig. 4). Anti-asialo GM1 was the only antiserum that significantly reduced the activity in each assay, establishing that the cells were Thy-1 and immunoglobulin negative, and asialo GM1 positive.

DISCUSSION

Recently, reports have indicated that NK cells are effective against target cells of nontissue origin (14, 37). The data presented here support that concept by demonstrating that murine splenic cells with characteristics of NK cells can inhibit the in vitro growth of *P. brasiliensis* yeast phase targets.

The initial approach taken in this study was to correlate levels of NK activity of various effector cell populations with the ability of the cells to inhibit the in vitro growth of *P. brasiliensis* yeast phase targets. No matter whether we varied the NK reactivity through enrichment by passage over NWC to remove the macrophages and B cells (Table 1) or by collecting effector cells from strains of mice with

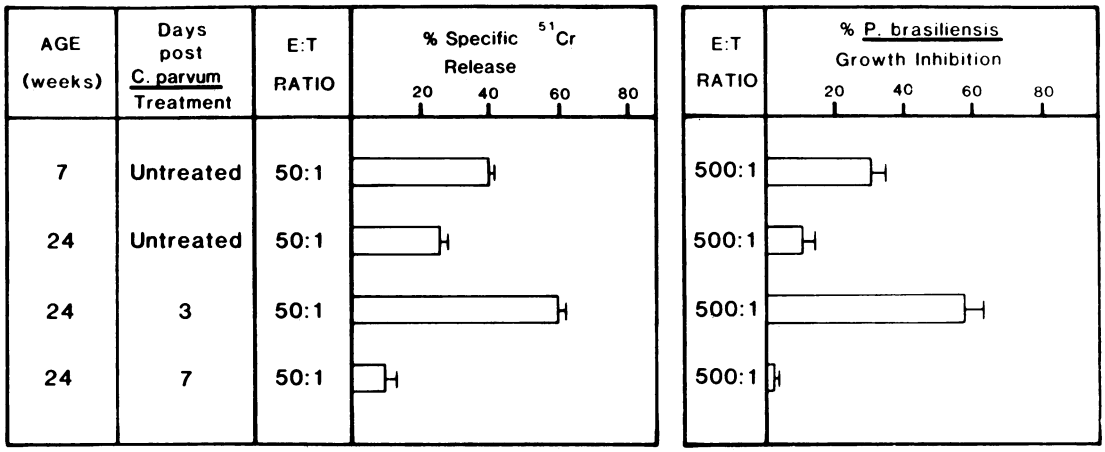

FIG. 3. Effects of injecting 24-week-old CBA/N mice with 0.7 mg of *C. parvum* at 3 or 7 days before NWN splenic cells were collected for use as effector cells in the 4-h ^{51}Cr release assay and the *P. brasiliensis* growth inhibition assay. NWN splenic cells from 7-week-old CBA/N mice were used as positive controls, and untreated 24-week-old CBA/N mice were included to obtain the NK activity for animals of that age. Bars designate the mean and SEM based on four experiments.

varying NK activity (Fig. 1), from mice of various ages (Table 3), or from mice treated with NK-augmenting and -depressing agents (Fig. 3), we found that in every case the NK activity correlated directly with the in vitro inhibitory ability against *P. brasiliensis* targets. These data provided indirect evidence which suggests that NK cells could effectively inhibit the in vitro growth of *P. brasiliensis*.

Murine NK cells characteristically are NWN cells with no immunoglobulin, low-density Thy-1 (17), and reasonably high levels of asialo GM1 antigen (22) on their surfaces; therefore, effector cells in these studies were analyzed for those characteristics. Since the spleen cell pools used as effector cells in the assays contained many different cell types, we began by eliminating certain cell populations. First, the spleen cells were passed over NWC (Table 1), and we found that NWN fractions which were depleted of macrophages and B cells had enhanced activities in the assay for NK activity and for *P. brasiliensis* growth-inhibiting activity. This was good evidence that the effector cells were not likely macrophages or B lymphocytes. Pretreatment of NWN splenic cells with anti-mouse immunoglobulin and complement before using the cells in the two assays did not eliminate the activities; this finding confirmed that B cells were not the effector cells. The possibility that T lymphocytes were the effector cells was eliminated because removal of T cells by treatment with anti-Thy-1 plus complement did not abolish the activities (Fig. 4). In contrast, when anti-asialo GM1, an antibody directed against a glycolipid on the surface of NK cells plus complement was used, the activities in both the ^{51}Cr release and *P. brasiliensis* growth inhibition assays were significantly reduced, demonstrating the presence of significant concentrations of asialo GM1 antigen on the effector cells. Some macrophages have been reported to have asialo GM1 on their surface in low density (19); however, at the dilution at which we used the anti-asialo GM1, the macrophages should not have been affected (23).

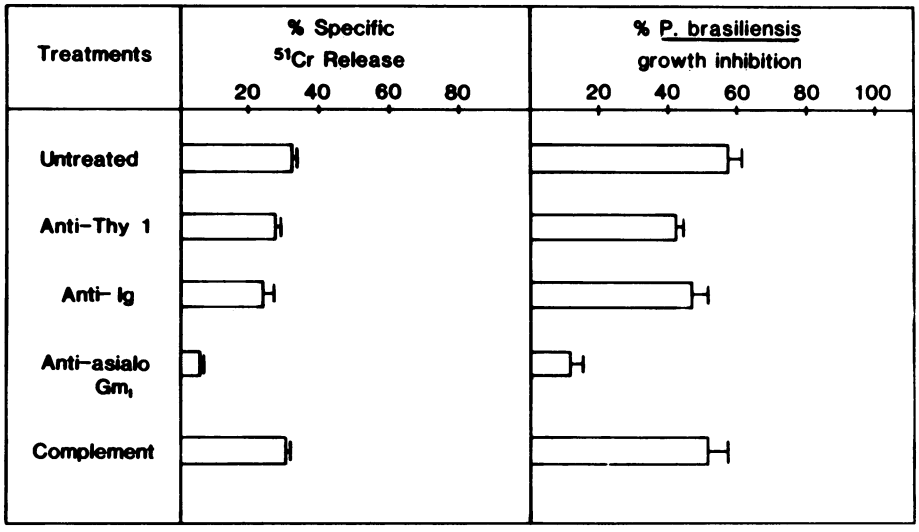

FIG. 4. Characterization of effector cells responsible for ^{51}Cr release from YAC-1 targets and for the inhibition of *P. brasiliensis* growth. NWN splenic cells from 7-week-old CBA/N mice were used as effector cells, either untreated or after being treated with anti-Thy-1, anti-mouse immunoglobulin, or anti-asialo GM1 and complement. Bars indicate the mean and SEM based on three experiments.

Furthermore, since macrophages had been eliminated from the cell pools by passage over NWC (Table 1), the reduction in activity by treatment of the effector cells with anti-asialo GM1 and complement was most likely due to elimination of NK cells. These data demonstrated that the effector cells in the growth inhibition assay against *Paracoccidioides* were phenotypically similar to the effector cells in the NK activity assay, adding more evidence that NK cells were responsible for *P. brasiliensis* growth inhibition.

Fractionation of mouse splenic cells by discontinuous Percoll density gradients clearly demonstrated an enrichment of NK activity and of *P. brasiliensis* growth-inhibiting activity in the low-density fractions (Table 2). With these purified cell populations, the effector-target (E:T) ratios could be reduced to as low as 100:1, and a substantial inhibition of *Paracoccidioides* growth could be obtained. Morphologically, the cells in the low-density fractions were similar to the large granular cells described by others (26, 30, 54). When fractions from parallel gradients were examined for their relative percentage of cells with immunoglobulin, Thy-1, and asialo GM1 on their surfaces or for staining with nonspecific esterase, we confirmed that the surface markers and staining characteristics of cells from fractions 1 and 2 were the same as those described for NK cells (Nabavi, unpublished data; Murphy, in press). As the effector cells were enriched for NK activity, by first passage over NWC and then by Percoll fractionation, we demonstrated that the correlation coefficients increased, indicating that effector cells in the growth inhibition assay were either NK cells or were another population of natural effector cells found concomitantly with NK cells and with similar levels of activity. The effector cells capable of inhibiting the growth of *P. brasiliensis* not only have activities that correlate the NK activities under many different circumstances, but they are morphologically and phenotypically similar to NK cells. Taken together, these data provide a strong argument for NK cells being reactive against *P. brasiliensis*.

The growth-inhibiting ability of natural effector cells was not unique for the LA isolate of *P. brasiliensis* (Fig. 2). In fact, effector cell pools with high NK activity were also more effective than were low-NK-reactive cell pools in inhibiting the growth of six other isolates of *P. brasiliensis* subjected to the growth inhibition assay. Some variation in susceptibility to inhibition from one *P. brasiliensis* isolate to another was observed; however, considerably more work is required to demonstrate that certain strains are consistantly more susceptible to inhibition than others.

In paracoccidioidomycosis, it is thought that the organism enters the host through the lungs, and generally a long incubation period ensues in humans (8) and experimental animals (20, 27). Since Puccetti et al. (43) have shown comparable levels of NK activity in the lungs and spleens of mice, it could be assumed that there is significant NK activity in human lungs. Therefore, one may speculate that NK cells could play a role in limiting the growth of *P. brasiliensis* in the lungs; thereby they aid in confining the disease to the lungs and minimizing dissemination to other tissues. Considerably more work is required to establish the effects of NK cells in vivo against *P. brasiliensis*; furthermore, it may be that NK cells function in vivo in concert with other cells, such as polymorphonuclear lymphocytes and macrophages, in eliminating *P. brasiliensis* cells from tissues before acquired immune resistance mechanisms begin to function.

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