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Correlation of Natural Killer Cell Activity and Clearance of *Cryptococcus neoformans* from Mice after Adoptive Transfer of Splenic Nylon Wool-Nonadherent Cells

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Previous reports demonstrate that natural killer (NK) cells inhibit the growth of *Cryptococcus neoformans* in vitro, but conclusive evidence supporting the effectiveness of NK cells in host resistance to cryptococci is not available. The objective of these studies was to assess the ability of NK cells to clear *C. neoformans* from the lungs, livers, and spleens of infected mice. CBA/J mice were depleted of NK cells, as well as other natural effector cells, by an intraperitoneal injection of cyclophosphamide (Cy), 240 mg/kg of body weight. One day later, 7.5×10^7 nylon wool-nonadherent (NWN) spleen cells, either untreated or treated with anti-asialo GM_1 and complement to remove NK cells, were adoptively transferred to Cy-pretreated mice. On day 2 after Cy treatment, the mice were injected intravenously with 2×10^4 cryptococci. At 4 and 6 days after Cy treatment, tissues were assayed for NK reactivity, using a 4-h ^{51}Cr-release assay, and for in vivo clearance of cryptococci as reflected by mean $\log_{10}$ CFU per organ. We observed that Cy treatment depleted NK activity against YAC-1 targets and reduced in vivo clearance of *C. neoformans* from the tissues of infected mice. Additionally, Cy treatment depleted the total lung and spleen cellularity and the total number of peripheral blood lymphocytes when compared with those in normal untreated control mice. Also, spleen weights were significantly decreased in comparison with those of untreated animals 4 days after Cy treatment. Adoptive transfer of untreated NWN spleen cells into Cy-depressed mice restored the NK cell activity which correlated with enhanced clearance of cryptococci from lungs, livers, and spleens. In contrast, treatment of NWN spleen cells with anti-asialo GM_1 and complement before adoptive transfer abrogated the ability of these cells to restore NK activity or reduce the numbers of cryptococci present in tissues of infected mice. Taken together, these data indicate that NK cells are the cells effective in diminishing the numbers of cryptococci in tissues of infected mice. Consequently, NK cells may play a role in first-line host resistance against *C. neoformans*.

Cryptococcus neoformans is a pathogenic, encapsulated, yeastlike organism with a predilection for the central nervous system. The organism is ubiquitous, and therefore exposure to the airborne infectious particles is common. Human infection usually occurs via the pulmonary route resulting in a subclinical respiratory infection which is often spontaneously resolved by the host. In approximately 10% of the individuals with the pulmonary disease, the organism becomes bloodborne, causing disseminated cryptococcosis, generally manifest as meningitis. Dissemination occurs most frequently in patients with impaired host resistance. Since the incidence of dissemination is lower than expected based on the frequency of exposure, we questioned the role of innate resistance in host defense against *C. neoformans*.

Generally, polymorphonuclear leukocytes (PMN) and macrophages are considered responsible for natural resistance to *C. neoformans* (8, 11, 28, 49). Recent studies by Murphy and McDaniel (36) demonstrate that a murine effector cell population having the characteristics of natural killer (NK) cells is capable of inhibiting the growth of cryptococci in vitro. This observation stimulated us to assess the in vivo effectiveness of NK cells in host resistance to *C. neoformans*.

NK cells were first recognized in the mid-1970s and have been found in the lymphoid tissues, except the thymus, of nearly all mammalian and avian species studied (19, 20, 22). In mice, NK cell activity is absent at birth, appears from 3 to 5 weeks of age, peaks from 6 to 8 weeks of age, and then declines to low levels (21, 27, 51). NK cells are characterized as being nonphagocytic and nonadherent (20) large granular lymphocytes (LGL) (30, 41, 50) with insignificant amounts of surface immunoglobulin, Thy-1, and Ia antigens (20). Asialo GM_1, a glycosphingolipid, is a characteristic antigenic surface marker present in relatively high density on NK cells (26, 29). A considerable amount of work has been done concerning the effectiveness of NK cells against various targets including tumor cells (1, 2, 16, 38), virus-infected cells (3, 5, 15, 46, 52), fungal pathogens (4, 24, 36), parasites (17), and bacteria (37).

Two earlier studies, using different models of murine cryptococcosis, present data that suggest that NK cells are effective in clearance of cryptococci from host tissues. First, Cauley and Murphy (9) reported that homozygous *nu/nu* mice clear cryptococci from the tissues more effectively early after infection than do heterozygous *nu/+* mice (35). Since this difference was observed early after infection and in mice incapable of developing a cell-mediated response, they concluded that an innate mechanism was functioning such as the nude mouse's highly activated macrophage system (10) or augmented NK cell populations (18) or both. Since data are contradictory concerning the abilities of macrophages to kill *C. neoformans* in vitro (11, 13, 28, 33, 48) and NK cells are quite effective in inhibiting the growth of cryptococci in vitro (36), the NK cells were likely to be, at least partially, responsible for the early clearance of cryptococci observed in the nude mouse.

In a second study, 3 days after infection with cryptococci, lungs and spleens of C57BL/6 *bg/bg* mice contained signifi-

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cantly greater numbers of cryptococci than the corresponding tissues from infected *bg*/+ littermates (35). The beige mutation in the mouse leads to low levels of NK cell activity in homozygous *bg*/*bg* mice without affecting killing functions of promonocytes and macrophages, whereas heterozygous *bg*/+ mice have normal levels of NK cell activity (43, 44). Considering these findings, one can postulate that NK cells were responsible for the clearance of *C. neoformans* from *bg*/+ animals.

The objective of the present study was to more clearly define the effectiveness of NK cells in the in vivo clearance of *C. neoformans* from the tissues of infected mice. To do this, mice were pretreated with cyclophosphamide (Cy) which depleted most of the natural effector cells, and then the animals were reconstituted with NK cell-enriched or NK cell-depleted spleen cells from syngeneic mice 1 day before infection with 2×10^4 cryptococci. Cy treatment depleted NK cell activity against YAC-1 targets and reduced clearance of *C. neoformans* from the tissues of infected mice. Additionally, Cy treatment depleted the total lung and spleen cellularity, the total number of peripheral blood lymphocytes, and spleen weights when compared with untreated controls. Adoptive transfer of NK cell-enriched populations reconstituted NK activity in various organs and enhanced clearance of cryptococci from the tissues of Cy-pretreated mice; whereas the transfer of NK cell-depleted populations did not restore NK activity in the tissues nor enhance clearance of cryptococci over that observed in Cy-pretreated infected animals.

MATERIALS AND METHODS

Mice. Female CBA/J mice, 5 weeks old, were obtained from Jackson Laboratory (Bar Harbor, Maine). These mice were maintained in the University of Oklahoma animal facility until use at 7 to 8 weeks of age.

Organism. *C. neoformans* 184A, a weakly encapsulated serotype A isolate (36), was maintained on modified Sabouraud agar slants. After 3 days of growth at 25°C, yeast cells were harvested in sterile physiological saline solution (SPSS), washed three times in SPSS, and adjusted to 2×10^5 cells per ml in SPSS. For clearance studies, 7- to 8-week-old mice were injected intravenously (i.v.) with 2×10^4 cryptococci. The number of *C. neoformans* injected was based on hemacytometer counts and confirmed by plate counts.

Peripheral blood analysis. Mice were injected intraperitoneally with 240 mg of Cy per kg of body weight in 0.1 ml of SPSS. At 4 and 6 days after treatment, animals were bled from the retro-orbital plexus into heparinized tubes (12). Whole blood was diluted with Unopette Platelet/WBC Reagent (Becton Dickinson and Co., Rutherford, N.Y.) in a Thoma leukocyte (WBC) diluting pipette (Dade Diagnostics Inc., Miami, Fla.) and the total numbers of WBC per mm^3 were determined by hemacytometer count. Differential peripheral blood counts were performed after diluting whole blood 1:200 in RPMI 1640 medium plus 10% fetal calf serum (FCS) and applying 200 μl of this suspension to a slide by using a cytocentrifuge. The hematological smears were stained with Diff-Quick (American Scientific Products, McGaw Park, Ill.), a modified Wright-Giemsa stain. The percentages of neutrophils, lymphocytes, and monocytes in 200 cells were determined by light microscopy.

Preparation of cells used for adoptive transfer. Normal mice were sacrificed by cervical dislocation. Spleens were excised and placed in separate sterile laboratory bags (Tekmar, Cincinnati, Ohio) containing 5 ml of RPMI 1640 medium. Single-cell suspensions were prepared by homogenizing the tissue in a Stomacher-80 laboratory blender (Tekmar, Cincinnati, Ohio) for 3 min. Erythrocytes were removed from spleen cell pools by treatment with Tris-ammonium chloride buffer (pH 7.2) (32). After two washes with Hanks balanced salt solution (HBSS) (GIBCO Laboratories, Grand Island, N.Y.), the spleen cell pellet was suspended in RPMI 1640 medium plus 5% FCS, and nylon wool-nonadherent cells (NWN) were collected as previously described (25). Approximately 20 to 30% of the original number of cells placed on a 1.4-g nylon wool column were routinely recovered in the nonadherent fraction. NWN spleen cells were adjusted to 7.5×10^7 cells per ml in RPMI 1640 medium for use as donor cells in the adoptive transfer studies. The NWN spleen cells were enriched for NK activity as shown by the 30 ± 0.4% lysis in a 4-h ^{51}Cr-release assay when compared with the 19.0 ± 1.0% lysis by the unfractionated spleen cells.

In vitro cytolytic assay. To define the level of NK activity in the various tissues, we performed a standard assay measuring ^{51}Cr released from YAC-1 target cells as previously described (24, 27, 36). YAC-1 cells were maintained in RPMI 1640 medium (GIBCO) supplemented with 10% heat-inactivated FCS, 100 U of penicillin per ml, and 100 μg of streptomycin (GIBCO) per ml. For the assay, the YAC-1 cells were labeled with 100 μCi of radioactive sodium chromate (New England Nuclear Corp., Boston, Mass.) by incubation for 1 h at 37°C in 5% CO_2. The labeled cells were washed to remove excess ^{51}Cr and adjusted to 2×10^5 cells per ml in RPMI 1640 containing 10% FCS for use as target cells. To assess NK activity of spleens, the NWN cell fractions were collected as described above and used as the effector cells.

To determine the NK activities in the lungs, we prepared single-cell suspensions from lungs by homogenizing the tissue in a Stomacher-80 laboratory blender for 3 min with 10 ml of prewarmed collagenase solution (120 U of type I collagenase; Sigma Chemical Co., St. Louis, Mo.) (47). After an additional 90-min incubation at 37°C, the lung cells were washed twice in HBSS. Erythrocytes were removed from the lung cell pools by treatment with Tris-ammonium chloride buffer (32). After two additional washes with HBSS, the lung cells were suspended in RPMI 1640 medium plus 5% FCS. The NWN lung cell fractions were collected as previously described (25) and used as effector cells in the ^{51}Cr-release assay.

For determining NK activity in the livers, we isolated effector cells by a modification of the method of Wiltrout et al. (54). Single-cell suspensions were prepared by gently mincing livers through a 200-mesh stainless screen into HBSS. After centrifugation for 10 min at $600 \times g$, erythrocytes were removed by treatment with Tris-ammonium chloride buffer (32). Prewarmed enzyme solution (10 ml) containing 0.05% (wt/vol) type II collagenase, 500 U of type I DNase (Sigma) per ml, and 5% FCS was added per ml of liver extract. After incubation at 37°C for 10 min with constant agitation, the digest was washed twice in cold Ca^{2+}- and Mg^{2+}-free HBSS (GIBCO), suspended in 3 to 5 ml of 30% (wt/vol) metrizamide (Sigma) in HBSS to a final ratio of 7 parts metrizamide to 5 parts packed liver digest, and then overlaid with 1.5 ml of phosphate-buffered saline. After centrifugation at $1,400 \times g$ for 20 min at 4°C, the cell layer at the phosphate-buffered saline-metrizamide interface was removed and washed twice in HBSS before being used as effector cells in the ^{51}Cr-release assay.

For the 4-h ^{51}Cr-release assay, effector cells from spleens, lungs, and livers were adjusted to 10^7 cells per ml in RPMI 1640 medium plus 10% FCS and dispensed in 0.1-ml volumes to quadruplicate wells of a round-bottom 96-well microtiter plate (Linbro Scientific Co., Hamden, Conn.). ^{51}Cr-labeled YAC-1 target cells were added to each well in a 0.1-ml volume. An effector cell/target cell ratio of 50:1 was routinely used in the 4-h ^{51}Cr-release assay. After incubation of the plates for 4 h at 37°C in 5% CO_2 and centrifugation for 10 min at 200 × *g*, 0.1 ml of supernatant was removed from each well and counted in a gamma counter (Beckman Instruments, Inc., Fullerton, Calif.) for 10 min. Spontaneous release was determined by counting the activity in the supernatants from wells containing 0.1 ml of labeled target cells and an equal volume of RPMI 1640 plus 10% FCS. Maximum release was determined from counts obtained from supernatants of wells containing 0.1 ml of labeled target cells and an equal volume of 2 N HCl. The percentage of ^{51}Cr released from YAC-1 targets was calculated by the following formula: percent ^{51}Cr release = [(experimental release − spontaneous release)/maximum release] × 100. Previous studies by us (24, 35, 36) as well as others (2, 7, 17, 27, 30, 37, 41–44, 47, 54) have demonstrated that the percentage of ^{51}Cr release is a direct correlate of the percentage of cytotoxicity or percentage of lysis of YAC-1 targets.

Antiserum treatment of cells. Anti-asialo GM_1 (Wako Pure Chemical Industries, Dallas, Tex.) has been used successfully to deplete NK cell activity in vivo and in vitro (1, 2, 16, 24, 38). To remove NK cells in the present study, 5×10^7 NWN spleen cells were suspended in 1 ml of anti-asialo GM_1 at a 1:40 dilution in HBSS and incubated for 30 min at 25°C with constant agitation. After incubation, the cells were washed with HBSS, suspended in 1 ml of agarose-absorbed guinea pig serum at a 1:10 dilution in HBSS as a source of complement (31), and incubated for 45 min at 37°C in 5% CO_2 with constant agitation. After washing three times with HBSS, the cells were adjusted to 7.5×10^7 cells per ml in RPMI 1640 medium for use in the adoptive transfer studies.

In vivo clearance assay. Mice were injected intraperitoneally with 240 mg of Cy (Sigma) per kg of body weight on day 0. The Cy-treated mice were divided into three groups, and 1 day after Cy treatment, group 1 mice were not treated, group 2 animals were injected i.v. with 7.5×10^7 NWN spleen cells (Cy + NWN), and group 3 mice were injected i.v. with 7.5×10^7 NWN spleen cells which had been treated in vitro with anti-asialo GM_1 and C to destroy the NK cells (Cy + [NWN + anti-asialo GM_1 + C]). On day 2 after Cy treatment, all groups, including age- and sex-matched untreated controls, were injected i.v. with 2×10^4 *C. neoformans* cells. Two and four days after injection of the cryptococci, three animals were sacrificed from each group to determine the number of CFU per organ. Lungs, livers, and spleens were excised, transferred to separate sterile Stomacher laboratory bags containing 5 ml of SPSS, and homogenized in the Stomacher-80 laboratory blender for 3 min. In selected experiments, the individual organs were weighed before homogenization. Each organ homogenate was appropriately diluted and plated in duplicate on modified Sabouraud agar plates. After a 72-h incubation at 25°C, CFU were enumerated. The arithmetic mean of the number of CFU per organ was determined at each time period.

Statistical analysis. Means, standard error of the means, and two-tailed Student's *t* tests were used to analyze the data.

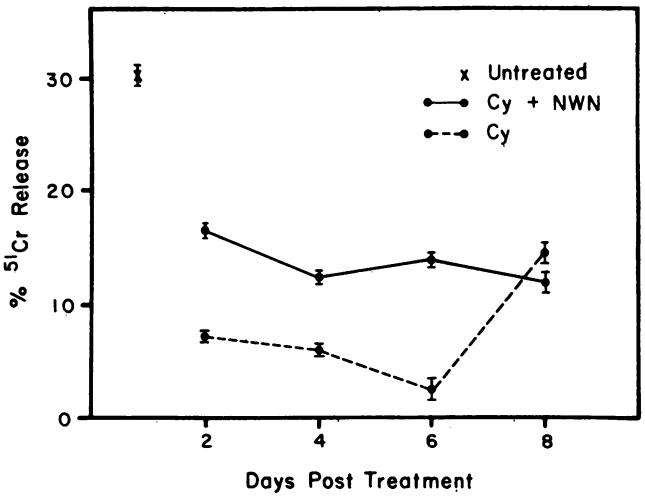

FIG. 1. NK activity, based on a 4-h ^{51}Cr-release assay, of NWN spleen cells from mice treated with Cy or Cy followed by reconstitution with NWN (NK cell-enriched) spleen cells. Assays were performed 2, 4, 6, and 8 days after Cy treatment. Untreated CBA/J animals were used as a control. Points designate the mean and standard error of the mean (bars) based on quadruplicate measurements.

RESULTS

Effects of Cy treatment and reconstitution with NWN spleen cells on in vitro NK reactivity. Cy, in appropriate doses, depletes NK cell, PMN, and macrophage function in vitro and in vivo (6, 23, 42). In a preliminary experiment, we determined the duration of the depletion of NK cell reactivity after Cy treatment and the effectiveness of restoring NK cell reactivity by adoptively transferring NWN spleen cells to Cy-pretreated mice. For these studies, mice were injected intraperitoneally with 240 mg of Cy per kg of body weight; then 1 day later one group of Cy-treated animals was given 7.5×10^7 syngeneic NWN spleen cells i.v. and one group received no cells. The NK activity of the splenic NWN cell pools used for the adoptive transfers was relatively high as indicated by the 30.0 ± 0.4% lysis of YAC-1 targets. To determine the level of NK reactivity restored by adoptive transfer of splenic NWN cells, 2, 4, 6, and 8 days after Cy treatment, the 4-h ^{51}Cr-release assay was performed, using as effector cells splenic NWN cells from normal age-matched mice, Cy-treated mice, or Cy-treated mice that had received splenic NWN cells (Cy + NWN). Data shown in Fig. 1 demonstrate that Cy significantly depleted splenic NK cell reactivity throughout the 8-day experimental period. NK levels in Cy-treated mice were significantly suppressed when compared with those of untreated normal controls ($P < 0.001$). Adoptive transfer of 7.5×10^7 splenic NWN cells to Cy-pretreated mice partially restored splenic NK cell activity. Although the restoration of NK activity was not complete, at 2, 4, and 6 days after Cy treatment, the mice given NWN cells had significantly higher levels of NK cell activity than did the mice treated with Cy but not reconstituted ($P < 0.001$). However, by 8 days post-Cy treatment, NK reactivity was rising in the Cy-treated mice, and there was not a significant difference in NK activities of Cy-treated and Cy + NWN-treated mice. Therefore, subsequent experiments were performed no later than 6 days after Cy treatment.

Organ cell yields and peripheral blood analysis. A pronounced splenic atrophy was observed in the Cy-treated

TABLE 1. Comparison of the total number of NWN cells collected per spleen and lungs 4 and 6 days after treatment of CBA/J mice with Cy, Cy followed by reconstitution with NWN spleen cells, or Cy followed by reconstitution with anti-asialo GM_1-pretreated NWN spleen cells[a]

Organ	Treatment	Total NWN cells, 10^6/organ ± SEM	
		Day 4	Day 6
Spleen	None	42 ± 8	40 ± 6
	Cy	3 ± 1	10 ± 3
	Cy + NWN	8 ± 2	49 ± 6
	Cy + (NWN + anti-asialo GM_1 + C)	5 ± 1	20 ± 5
Lungs	None	1.4 ± 0.3	1.9 ± 0.9
	Cy	0.5 ± 0.2	1.4 ± 0.3
	Cy + NWN	0.9 ± 0.2	2.4 ± 0.8
	Cy + (NWN + anti-asialo GM_1 + C)	0.7 ± 0.1	1.0 ± 0.2

[a] Untreated normal mice were used as a control. The means and standard error of the means were based on data from eight animals.

mice and in the Cy-treated animals that had received NWN spleen cells which had been treated in vitro with anti-asialo GM_1 and C (Cy + [NWN + anti-asialo GM_1 + C]), whereas the spleens of Cy + NWN-treated mice were similar in size to those of untreated animals. Other organs did not display atrophy after Cy treatment. Data in Table 1 demonstrate the decrease in the total yield of NWN cells per lungs and spleen 4 and 6 days after Cy treatment. The numbers of splenic NWN cells were reduced 93% in Cy-treated mice when compared with untreated normal controls. In the spleens of Cy-treated mice and Cy + [NWN + anti-asialo GM_1 + C]-treated mice, there was a 70% ($P < 0.05$) and 50% ($P < 0.02$) reduction in the numbers of NWN cells, respectively, when compared with the numbers of splenic NWN cells from the Cy + NWN-treated group. The numbers of lung NWN cells were reduced 64% after Cy treatment in comparison with untreated normal controls. In the lungs, a 30 to 50% reduction in NWN cells was observed in the Cy-treated and Cy + [NWN + anti-asialo GM_1 + C]-treated groups as compared with the numbers of NWN lung cells from the Cy + NWN-treated group.

Data in Table 2 illustrate the effect of Cy treatment upon the presence of several cell types involved in natural resistance. We observed a 98% reduction ($P < 0.001$) in the total numbers of WBC present in the peripheral blood of mice 4 days after Cy treatment when compared with normal mice. The remaining cells in the Cy-treated mice were predominantly lymphocytes (93.0 ± 2.0%). However, the absolute numbers of lymphocytes present in Cy-treated animals (140 ± 20/mm^3) were 98% less than the absolute numbers of lymphocytes found in untreated animals (3,690 ± 190/mm^3). A 67% reduction ($P < 0.001$) in total WBC was maintained over a 6-day period after Cy treatment. At this later time, PMN predominated (79.0 ± 3.1%); however, they exhibited juvenile morphology, appearing large with banded nuclei. This was in contrast to PMN from untreated mice which appeared morphologically mature and were small with typical segmented nuclei. If the juvenile PMN are included, the absolute numbers of PMN present in Cy-treated mice (1,530 ± 340/mm^3) were not significantly different from the absolute numbers of PMN found in untreated animals (1,590 ± 80/mm^3) at this time.

Comparison of NK cell reactivity of lungs, livers, and spleens with in vivo clearance of *C. neoformans* from the respective organs. On the basis of the ability to deplete and restore NK cell activity in vivo, we sought to compare the NK cell reactivity of lungs, livers, and spleens with the clearance of viable *C. neoformans* from the corresponding organs. On day 0, mice were treated with Cy as before (240 mg/kg). The Cy-treated animals were divided into three groups, and an untreated, age-matched control group was included. One day after Cy treatment, one group of Cy-treated mice was reconstituted with splenic NWN cells (Cy + NWN), and another group of Cy-treated animals was injected with splenic NWN cells which had been previously treated in vitro with anti-asialo GM_1 and C to remove NK cells (Cy + [NWN + asialo GM_1 + C]). Two days after Cy treatment, all four groups, i.e., untreated, Cy, Cy + NWN, and Cy + [NWN + anti-asialo GM_1 + C], were injected i.v. with 2×10^4 *C. neoformans* cells. At 4 and 6 days post-Cy treatment, or days 2 and 4 postinfection, respectively, three mice from each of the four groups were sacrificed, and the numbers of cryptococci CFU per lung, liver, and spleen were determined. Simultaneously, 4-h ^{51}Cr-release assays were performed to determine the NK reactivities in lungs and spleens of mice from the various treatment groups. The splenic NWN cells used for adoptive transfer had substantial NK activity as indicated by the 38.7 ± 0.8% lysis of YAC-1 targets in a 4-h ^{51}Cr-release assay, whereas the same splenic NWN cells treated with anti-asialo GM_1 and C had significantly reduced NK activity (0.4 ± 0.2% lysis of YAC-1 targets).

Lung NK reactivity was reduced 64% on day 4 and 89% on day 6 ($P < 0.001$) after Cy treatment when compared with the NK activity of normal untreated controls (Fig. 2). Comparisons of the numbers of cryptococci CFU per lungs of Cy-treated mice with the numbers of CFU per lungs of untreated controls at both 4- and 6-day periods showed that clearance of cryptococci from lungs of Cy-treated mice was significantly impaired ($P < 0.001$). Significantly higher NK reactivity was observed in the lungs of the mice reconstituted with splenic NWN cells (Cy + NWN) when compared with the NK reactivity in the lungs of the Cy-treated or the Cy + [NWN + asialo GM_1 + C]-treated groups (Fig. 2) ($P < 0.001$). By comparing the numbers of cryptococci CFU per lungs in the various groups, the Cy + NWN group showed

TABLE 2. Analysis of peripheral blood from CBA/J mice 4 and 6 days after Cy treatment[a]

Treatment	Total WBC (10^3/mm^3 ± SEM)	% Total WBC ± SEM (absolute cell no., 10^3/mm^3 ± SEM)		
		PMN	Lymphocytes	Monocytes
None	5.70 ± 0.42	27.9 ± 1.6 (1.59 ± 0.08)	64.6 ± 1.8 (3.69 ± 0.19)	7.0 ± 0.7 (0.40 ± 0.02)
Cy (4 days before)	0.15 ± 0.02	1.0 ± 1.0 (0.002 ± 0.003)	93.0 ± 2.0 (0.14 ± 0.02)	6.0 ± 1.0 (0.009 ± 0.001)
Cy (6 days before)	1.93 ± 0.37	79.0 ± 3.1 (1.53 ± 0.34)	6.6 ± 1.4 (0.13 ± 0.03)	14.2 ± 1.9 (0.27 ± 0.06)

[a] Normal CBA/J mice were used as a control. The means and standard error of the means were based on data from eight animals.

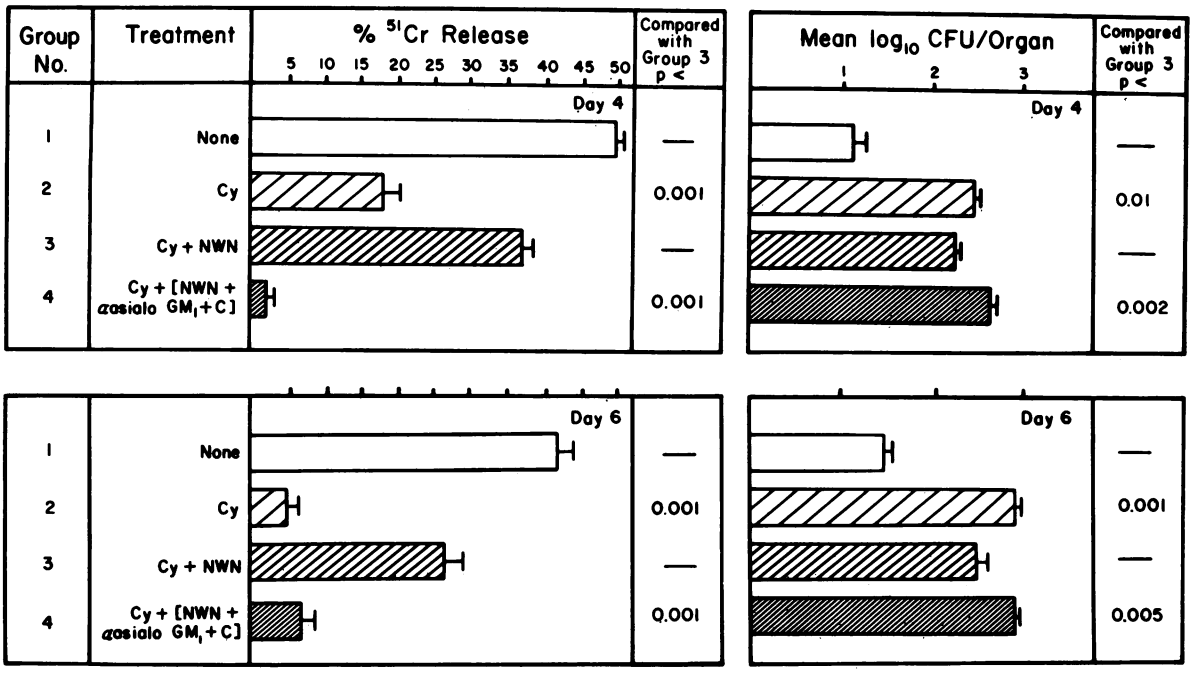

FIG. 2. Lung NK cell activities based on a 4-h ^{51}Cr-release assay and clearance of *C. neoformans* from lungs of normal CBA/J mice (group 1), Cy-treated mice (group 2), Cy-treated mice after adoptive transfer of NK cell-enriched NWN spleen cells (group 3), or Cy-treated mice after adoptive transfer of NK cell-depleted NWN spleen cells (group 4). Assays were performed 4 and 6 days after Cy treatment. Bars represent the mean and standard error of the mean representative of 4 experiments.

significantly better clearance of cryptococci from the lungs than either the Cy-treated group or the Cy + [NWN + asialo GM_1 + C]-treated group (Fig. 2). Similar results were obtained both 4 and 6 days after Cy treatment.

In contrast to lungs, livers from untreated mice had very low levels of NK reactivity, i.e., 8.6 ± 0.8% lysis of YAC-1 cells for livers as compared to 50.0 ± 1.0% lysis for lungs. Cy treatment of mice 6 days before the assay totally ablated NK reactivity in the livers. Owing to the difficulty in isolating sufficient numbers of liver NWN cells, the levels of NK reactivity in the livers were not determined in parallel with in vivo clearance studies in the liver. Figure 3 shows the numbers of cryptococcal CFU in livers 4 and 6 days after Cy treatment. At both times, the Cy + NWN-treated mice were significantly more effective in clearing cryptococci from the liver than either the Cy-treated group or the Cy + [NWN + anti-asialo GM_1 + C]-treated group.

Data shown in Fig. 4 demonstrate that splenic NK reactivity was significantly reduced 4 days (71%) and 6 days (88%) post-Cy treatment when compared with untreated normal animals ($P < 0.001$). However, when the numbers of cryptococcal CFU per spleen were compared between Cy-treated mice and normal untreated controls, significant differences in cryptococcal clearance were not obtained at either time (Fig. 4). On the basis of differences observed in NK reactivities between the untreated control and Cy-treated groups (Fig. 4) and our findings in lungs (Fig. 2), we had expected to find significantly impaired clearance of *C. neoformans* from spleens of Cy-treated animals when compared with untreated normal controls. Since this was not observed, we questioned how the severe splenic atrophy noted in the Cy-treated groups might affect the clearance of cryptococci. Spleens were the only organ in which a reduction in size was observed after Cy treatment. So, to take into consideration the differences in spleen size between the Cy-treated and untreated control groups, the data were reevaluated on the basis of numbers of cryptococcal CFU per gram of spleen weight rather than CFU per entire organ. When the CFU were expressed per gram of spleen weight, the untreated mice had 3.84 ± 0.05 $\log_{10}$ CFU in contrast to 4.32 ± 0.07 $\log_{10}$ CFU for the Cy-treated group 4 days after treatment ($P < 0.001$). These data indicate that clearance of cryptococci from spleens was significantly impaired in Cy-treated animals if spleen size was considered. Similar calculations were made for the data obtained 6 days after Cy treatment for the untreated and Cy-treated groups. The untreated group had 4.35 ± 0.06 $\log_{10}$ CFU/g of spleen, whereas the Cy-treated group had 4.47 ± 0.03 $\log_{10}$ CFU/g of tissue. However, considering the differences in spleen weights 6 days after Cy treatment, the clearance differences were only marginally significant ($P < 0.10$).

As observed in our preliminary study of Cy treatment and adoptive transfer (Fig. 1), splenic NK reactivity was significantly restored by adoptively transferring NWN cells to Cy-treated mice when compared with either the Cy-treated group or the Cy + [NWN + anti-asialo GM_1 + C]-treated group at both 4 and 6 days after Cy treatment (Fig. 4). When the numbers of cryptococcal CFU were calculated per entire organ, the spleens of the Cy + NWN group showed significantly enhanced clearance of cryptococci when compared with the spleens of the Cy + [NWN + anti-asialo GM_1 + C] group, 4 ($P < 0.02$) and 6 ($P < 0.001$) days post-Cy treatment (Fig. 4). Even though these calculations were based on the numbers of cryptococcal CFU per entire organ, significantly enhanced clearance of cryptococci was observed in the spleens of the Cy + NWN group when compared with the Cy + [NWN + anti-asialo GM_1 + C] group. A greater amount of splenic atrophy was observed in the Cy + [NWN + anti-asialo GM_1 + C] group than in the Cy + NWN group, indicating that the differences in clearance between the two

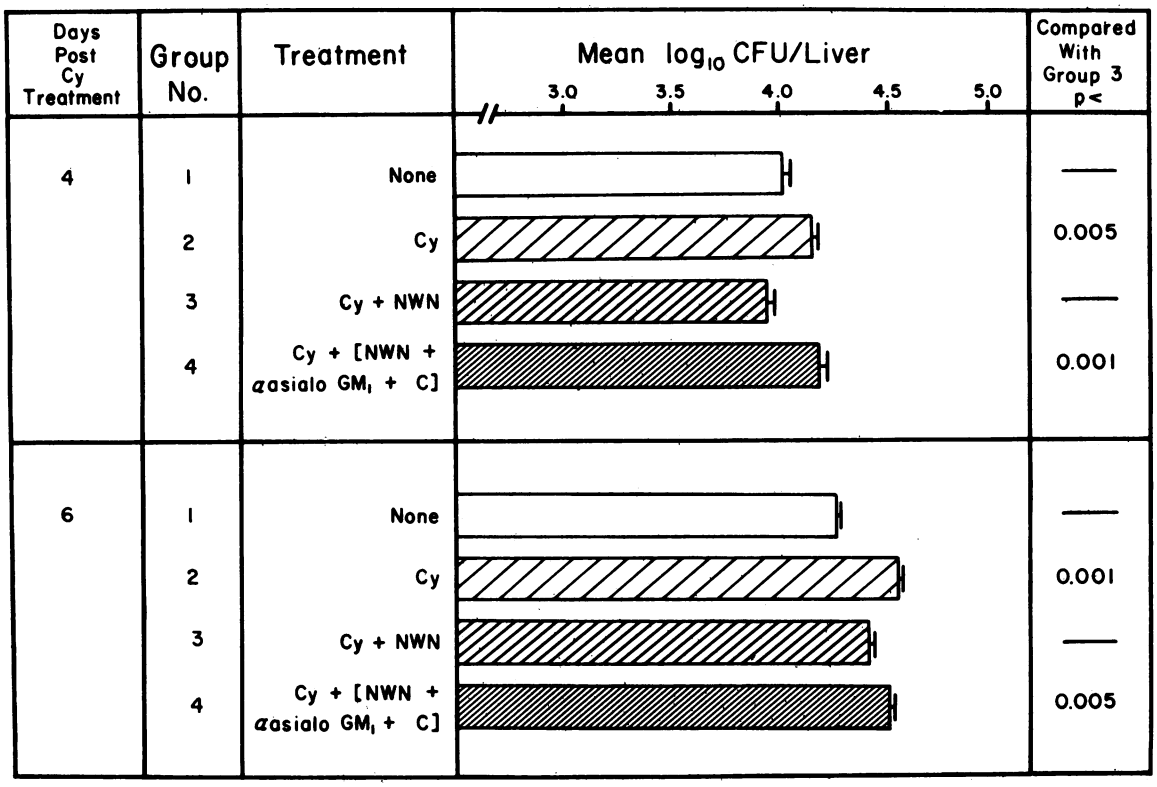

FIG. 3. Clearance of *C. neoformans* from livers of normal CBA/J mice (group 1), Cy-treated mice (group 2), Cy-treated mice after adoptive transfer of NK cell-enriched spleen cells (group 3), or Cy-treated mice after adoptive transfer of NK cell-depleted spleen cells (group 4). Assays were performed 4 and 6 days after Cy treatment. Bars designate the mean and standard error of the mean representative of four experiments.

groups would have been even greater had CFU per gram of spleen weight been used to compare the two groups rather than CFU per entire organ.

DISCUSSION

The experiments presented here were designed to assess the effects of adoptively transferred NK cell-enriched and NK cell-depleted populations on clearance of *C. neoformans* from lungs, livers, and spleens of Cy-treated mice over a period of 4 days after injecting the organism. For these studies, it was necessary to use a model in which natural effector cell functions could be selectively depressed and reconstituted. Cy is an alkylating agent capable of depressing many facets of natural and immune resistance (34, 39, 45). Several investigators have demonstrated the depletion of NK cell activities and numbers of PMN for proionged periods after Cy treatment (4, 23, 42). In our experiments, Cy treatment effectively depressed the NK cell activity in lungs (Fig. 2), livers (Fig. 3), and spleens (Figs. 1 and 4), the total yield of NWN cells per organ (Table 1), and the total number of WBC present in the peripheral blood through 6 days (Table 2). These results are in accordance with those of Riccardi et al. (42) who also show that NK cell activity in lungs and spleens remains depressed through 6 days after Cy administration (240 mg/kg). These combined data suggest that organs of Cy-treated mice are not naturally repopulated with NK cells during the first 6 days after Cy treatment, making Cy-treated mice an appropriate model for assessing the effects of various adoptively transferred cell populations on the clearance of cryptococci. The overall depression in the total number of WBC in peripheral blood in conjunction with the differential counts indicates that the absolute numbers of lymphocytes and monocytes were greatly reduced 4 and 6 days post-Cy treatment, whereas the absolute numbers of peripheral blood PMN were only significantly reduced 4 days after Cy treatment. When the juvenile forms of PMN were included in the differential counts, the absolute numbers of PMN were not significantly different in Cy-treated and untreated animals 6 days after Cy treatment. Therefore, the clearance of cryptococci attributed to the PMN and monocytes in the Cy-treated mice reconstituted with NWN spleen cells is minimal 4 days post-Cy treatment; however, the juvenile PMN could have had an effect on clearance of cryptococci 6 days after Cy treatment. Despite the presence of juvenile PMN in all Cy-treated mice 6 days after treatment, animals reconstituted with an NK cell-enriched population (Cy + NWN) showed significantly enhanced clearance of cryptococci when compared with Cy-treated or Cy + [NWN + anti-asialo GM_1 + C]-treated mice. These data suggest that the juvenile forms of PMN had a minimal effect on clearance of cryptococci from the tissues, indicating that the adoptively transferred NWN cells were responsible for the observed differences in clearance of cryptococci. Just as observed in this study (Table 2), other investigators have noted an early (3 to 6 day) decrease in spleen weight and cellularity and a significant reduction in total peripheral blood leukocytes through 6 days after Cy administration (4, 14). This model allowed us to effectively separate the role of NK cells in resistance to *C. neoformans* from the role of other natural effector cells depressed by Cy treatment.

Two separate populations of NK cells have been described in lungs (53) and livers (54), those which are associ-

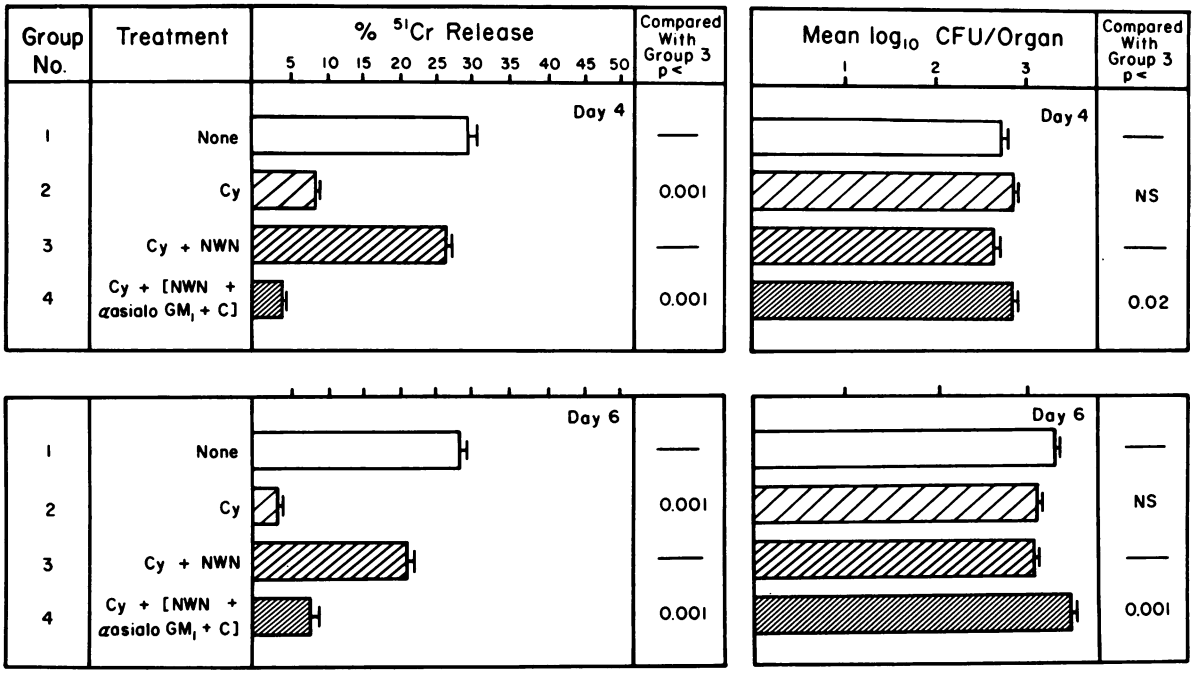

FIG. 4. Splenic NK cell activity based on a 4-h ^{51}Cr-release assay and clearance of *C. neoformans* from spleens of normal CBA/J mice (group 1), Cy-treated mice (group 2), Cy-treated mice after adoptive transfer of NK cell-enriched spleen cells (group 3), or Cy-treated mice after adoptive transfer of NK cell-depleted spleen cells (group 4). Assays were performed 4 and 6 days after Cy treatment. Bars represent the mean and standard error of the mean representative of four experiments.

ated with the peripheral blood supply of the organ and those which remain associated with tissue cells. Furthermore, organ-associated lung and liver NK cells were found to be more resistant to depletion by anti-asialo GM_1 treatment than blood and spleen NK cells (53). Our data suggest that Cy treatment depletes splenic NK cells more effectively than lung NK cells. The total numbers of splenic NWN cells were reduced 93% (Table 1) and splenic NK activity was reduced 71% (Fig. 4) 4 days after Cy treatment when compared with normal untreated controls. In contrast, the total numbers of lung NWN cells (Table 1) and lung NK activity (Fig. 2) were both reduced only 64% 4 days after Cy treatment when compared with normal untreated controls.

In the studies presented here, untreated mice had higher levels of NK activities in lungs (50.0 ± 1.0% lysis of YAC-1 cells) than in spleens (30.0 ± 0.4% lysis of YAC-1 cells) or livers (8.6 ± 0.8% lysis of YAC-1 cells). In addition, the level of lung NK activity of Cy-treated mice remained 53% higher than the level of splenic NK activity in the same mice. Furthermore, we found that 4 days after Cy treatment, lungs of Cy + NWN-reconstituted mice had a 30% higher level of NK reactivity than did the spleens of the same mice. However, by 6 days after Cy treatment, lungs of Cy + NWN-treated mice had only an 18% higher level of NK activity than spleens (Fig. 2 and 4). Reynolds and colleagues (40) have reported that within minutes after adoptive transfer of LGL into rats, the LGL are transiently located in the lungs. After several hours, decreasing numbers of lung LGL are accompanied by increasing numbers of spleen LGL (40). Our data indicate that levels of lung NK activity are initially higher and remain higher after Cy treatment than levels of splenic NK activity. Furthermore, NWN cells repopulated lungs earlier after adoptive transfer than they did spleens. These observations may account for the more pronounced clearance of cryptococci from the lungs than from the spleens of animals reconstituted with an NK cell-enriched population. This is an important observation since natural cryptococcal infections occur via the respiratory system.

The data in Fig. 2 to 4 demonstrate that the adoptive transfer of NK cell-enriched but not NK cell-depleted populations into mice depleted of natural effector cells enhances clearance of *C. neoformans*. Therefore, we concluded that NK cells are effective in vivo in reducing the numbers of cryptococci present in the tissues of infected mice. Furthermore, our data indicate that NK cells reduce cryptococci more effectively in the lungs than in the spleens. Other investigators have also shown that the adoptive transfer of NK cell-enriched spleen cell populations confers partial protection against murine cytomegalovirus (7) and enhances clearance of radiolabeled tumor cells (42). As in our model, protection conferred by adoptive transfer correlated with the level of NK reactivity of the donor cells (42).

Although data presented here as well as the studies reported by Cauley and Murphy (9) indicate that NK cells and other natural effector cells can reduce the numbers of cryptococci in the tissues of infected mice, the effectiveness of these cells is limited. In this study, the enhanced clearance of cryptococci from the tissues of Cy + NWN-treated mice when compared with Cy + [NWN + anti-asialo GM_1 + C]-treated animals was most likely due to NK cells, whereas in the studies of Cauley and Murphy, the enhanced clearance of cryptococci from the tissues of nude mice 7 days after infection could have been due to NK cells or activated macrophages.

Considering the results of all the in vivo clearance studies, it does not appear that NK cells alone can eliminate a *C. neoformans* burden as large as the one used in our experiments (2×10^4 cryptococci) but rather that the NK cells function early after infection to aid in reducing the numbers of cryptococci. This was apparent since after infecting mice

i.v. with 2×10^4 *C. neoformans*, we observed only a 37% of a log reduction in the numbers of cryptococci present in lungs of Cy + NWN-treated mice when compared with lungs of Cy + [NWN + anti-asialo GM_1 + C]-treated mice. However, this reduction was statistically significant ($P < 0.002$). Murphy reported similar results in the *bg/bg* = *bg/+* model. In that study, 39% of a log reduction in the numbers of cryptococci present in the lungs of *bg/+* mice was observed when compared with the lungs of *bg/bg* mice 3 days after i.v. infection with 2×10^4 *C. neoformans* cells (35). It must be considered that natural cryptococcal infections occur via the respiratory system, rather than i.v., and that the numbers of cryptococci which actually gain entrance into the lungs of the host are generally much lower than in our experimental model. Our data suggest that under natural conditions of exposure to cryptococci, NK cells may be quite effective in reducing or even eliminating the numbers of viable cryptococci in the lungs, whereas with a higher exposure to the organism, other host defense mechanisms are required to curtail the infection. Therefore, NK cells may serve as an effective first-line defense in the lungs where the numbers of organisms could be limited at the initiation of a natural infection.

Several separate murine models suggest that NK cells are effective in first-line host resistance to *C. neoformans* (35, 36). NK cells are capable of inhibiting the growth of *C. neoformans* in vitro (36). Also, enhanced clearance of *C. neoformans* early after infection is observed in the tissues of mice with high levels of NK activity when compared with the corresponding tissues of mice with low levels of NK activity in both the *bg/bg* = *bg/+* and nude mouse models (35). This study further emphasizes the effectiveness of NK cells in vivo against cryptococci by demonstrating that the adoptive transfer of NK cells into Cy-depressed mice restores NK reactivity which correlates with enhanced clearance of *C. neoformans* from the tissues. NK cells may be effective at inhibiting the initial cryptococcal infection in the lung, which might help to explain the observed low incidence of cryptococcosis despite frequent exposure to the organism. The adoptive transfer model defined here can be further utilized to study natural resistance mechanisms during cryptococcal infections.

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LITERATURE CITED

1. **Barlozzari, T., C. W. Reynolds, and R. B. Herberman.** 1983. Reconstitution of NK activity and antitumor immunity in anti-asialo GM_1-treated rats by adoptive transfer of LGL, p. 378–383. *In* T. Hoshino, H. S. Koren, and A. Uchida (ed.), NK activity and its regulation. International Congress Series 641. Excerpta Medica, Amsterdam.
2. **Barlozzari, T., C. W. Reynolds, and R. B. Herberman.** 1983. In vivo role of natural killer cells: involvement of large granular lymphocytes in the clearance of tumor cells in anti-asialo GM_1-treated rats. J. Immunol. **131**:1024–1027.
3. **Biron, C. A., and R. M. Welsh.** 1982. Activation and role of natural killer cells in virus infections. Med. Microbiol. Immunol. **170**:155–172.
4. **Bistoni, F., M. Baccarini, E. Blasi, P. Marconi, P. Puccetti, and E. Garaci.** 1983. Correlation between in vivo and in vitro studies of modulation of resistance to experimental *Candida albicans* infection by cyclophosphamide in mice. Infect. Immun. **40**:46–54.
5. **Bloom, B. R., N. Minato, A. Neighbor, and D. Marcus.** 1980. Interferon and NK cells in resistance to virus persistantly infected cells and tumors, p. 505–512. *In* R. B. Herberman (ed.), Natural cell-mediated immunity against tumors. Academic Press, Inc., New York.
6. **Bonavida, B., H. Kodo, M. Colvin, H. Brautbar, B. Gale, and L. T. Lebow.** 1983. Cyclophosphamide inhibits both the recognition and lethal hit stages of NK cell-mediated cytotoxicity, p. 283–288. *In* T. Hoshino, H. S. Koren, and A. Uchida (ed.), NK activity and its regulation. International Congress Series 641. Excerpta Medica, Amsterdam.
7. **Bukowski, J. F., J. F. Warner, G. Dennert, and R. M. Welsh.** 1985. Adoptive transfer studies demonstrating the antiviral effect of natural killer cells in vivo. J. Exp. Med. **161**:40–52.
8. **Bulmer, G. S., and J. R. Tacker.** 1975. Phagocytosis of *Cryptococcus neoformans* by alveolar macrophages. Infect. Immun. **11**:73–79.
9. **Cauley, L. K., and J. W. Murphy.** 1979. Response of congenitally athymic (nude) and phenotypically normal mice to *Cryptococcus neoformans* infection. Infect. Immun. **23**: 644–651.
10. **Cheers, C., and R. Waller.** 1975. Activated macrophages in congenitally athymic "nude" mice. J. Immunol. **115**:844–847.
11. **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**:367–375.
12. **Garvey, J. S., N. E. Cremer, and D. H. Sussdorf.** 1977. Methods in Immunology, p. 34–35. W. A. Benjamin, Inc., Reading, Mass.
13. **Gentry, L. O., and J. S. Remington.** 1971. Resistance against cryptococcus conferred by intracellular bacteria and protozoa. J. Infect. Dis. **123**:22–31.
14. **Graybill, J. R., and L. Mitchell.** 1978. Cyclophosphamide effects on murine cryptococcosis. Infect. Immun. **21**:674–677.
15. **Habu, S., K. Akamatsu, N. Tamaoki, and K. Okumura.** 1984. In vivo significance of NK cell on resistance against virus (HSV-1) infections in mice. J. Immunol. **133**:2743–2747.
16. **Habu, S., H. Fukui, K. Shimamura, M. Kasai, Y. Nagai, K. Okumura, and N. Tamoki.** 1981. In vivo effects of anti-asialo GM_1. I. Reduction of NK activity and enhancement of transplanted tumor growth in nude mice. J. Immunol. **127**:34–38.
17. **Hatcher, F. M., and R. E. Kuhn.** 1982. Destruction of *Trypanosoma cruzi* by natural killer cells. Science **218**:295–298.
18. **Herberman, R. B.** 1978. Natural cell-mediated cytotoxicity in nude mice, p. 135–136. *In* J. Fogh and G. C. Giovanella (ed.), The nude mouse in experimental and clinical research. Academic Press, Inc., New York.
19. **Herberman, R. B. (ed.).** 1980. Natural cell-mediated immunity against tumors. Academic Press, Inc., New York.
20. **Herberman, R. B., and H. T. Holden.** 1978. Natural cell-mediated immunity. Adv. Cancer Res. **27**:305–372.
21. **Herberman, R. B., M. E. Nunn, and D. H. Lavin.** 1975. Natural cytotoxic reactivity of mouse lymphoid cells against syngeneic and allogeneic tumors. I. Distribution of reactivity and specificity. Int. J. Cancer **16**:216–229.
22. **Herberman, R. B., and J. R. Ortaldo.** 1981. Natural killer cells: their role in defenses against disease. Science **24**:24–30.
23. **Hurtler, B., P. H. Lagrange, and J. C. Michel.** 1980. Systemic candidiasis in mice. II. Main role of polymorphonuclear leukocytes in resistance to infections. Ann. Immunol. (Paris) **131**:105–118.
24. **Jimenez, B. E., and J. W. Murphy.** 1984. In vitro effects of natural killer cells against *Paracoccidioides brasiliensis* yeast phase. Infect. Immun. **46**:552–558.
25. **Julius, M. H., E. Simpson, and L. A. Herzenberg.** 1973. A rapid method for isolation of functional thymus-derived murine lymphocytes. Eur. J. Immunol. **3**:645–649.
26. **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.
27. **Kiessling, R., E. Klein, and H. Wigzell.** 1975. "Natural" killer cells in the mouse. I. Cytotoxic cells with specificity for mouse

Moloney leukemia cells. Specificity and distribution according to genotype. Eur. J. Immunol. **5**:112–117.

28. **Kitz, D. J., C. R. Johnson, G. S. Kobayashi, G. Medoff, and J. R. Little.** 1984. Growth inhibition of *Cryptococcus neoformans* by cloned cultured murine macrophages. Cell. Immunol. **88**:489–500.
29. **Koo, G. C., J. B. Jacobson, G. J. Hammerling, and U. Hammerling.** 1980. Antigenic profile of murine natural killer cells. J. Immunol. **125**:1003–1006.
30. **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.
31. **Mishell, B. B., and S. M. Shiigi. (ed.).** 1980. Complement, p. 446–447. *In* Selected methods in cellular immunology. W. H. Freeman and Co., San Francisco.
32. **Mishell, B. B., S. M. Shiigi, C. Henry, E. L. Chan, J. North, R. Gallity, M. Slomick, K. Mieler, J. Marbrook, D. Parks, and A. H. Good.** 1980. Preparation of mouse cell suspensions, p. 3–27. *In* B. B. Mishell and S. M. Shiigi (ed.), Selected methods in cellular immunology. W. H. Freeman and Co., San Francisco.
33. **Mitchell, T. G., and L. Friedman.** 1972. In vitro phagocytosis and intracellular fate of variously encapsulated strains of *Cryptococcus neoformans*. Infect. Immun. **5**:491–498.
34. **Mitsuoka, A., M. Baba, and S. Morikawa.** 1976. Enhancement of delayed hypersensitivity by depletion of suppressor T-cells with cyclophosphamide in mice. Nature (London) **262**:77–78.
35. **Murphy, J. W.** 1982. Natural cell-mediated resistance in cryptococcosis, p. 1503–1511. *In* R. B. Herberman (ed.), NK cells and other natural effector cells. Academic Press, Inc., New York.
36. **Murphy, J. W., and D. O. McDaniel.** 1982. In vitro reactivity of natural killer (NK) cells against *Cryptococcus neoformans*. J. Immunol. **128**:1577–1583.
37. **Nencioni, L., L. Villa, D. Boraschi, B. Berti, and A. Taliabue.** 1983. Natural and antibody-dependent cell-mediated activity against *Salmonella typhimurium* by peripheral and intestinal lymphoid cells in mice. J. Immunol. **130**:903–907.
38. **Okumura, K., S. Habu, and M. Kasai.** 1982. The role of NK cells in resistance of in vivo tumors. Adv. Exp. Med. Biol. **155**:773–784.
39. **Ozer, H., J. W. Cowens, M. Colvin, A. Nussbaum-Blumenson, and D. Sheedy.** 1982. In vitro effects of 4-hydroperoxycyclophosphamide on human immuno-regulatory T subset function. I. Selective effects on lymphocyte function in T-B cell collaboration. J. Exp. Med. **155**:276–290.
40. **Reynolds, C. W., A. C. Denn, T. Barlozzari, R. H. Wiltrout, D. A. Reichardt, and R. B. Herberman.** 1984. Natural killer activity in the rat. IV. Distribution of large granular lymphocytes (LGL) following intravenous and intraperitoneal transfer. Cell. Immunol. **86**:371–380.
41. **Reynolds, C. W., T. Timonen, and R. B. Herberman.** 1981. Natural killer (NK) cell activity in the rat. I. Isolation and characterization of the effector cells. J. Immunol. **127**:282–287.
42. **Riccardi, C., T. Barlozarri, A. Santoni, R. B. Herberman, and C. Cesarini.** 1981. Transfer to cyclophosphamide treated mice of natural killer (NK) cells and in vivo natural activity against tumors. J. Immunol. **126**:1284–1289.
43. **Roder, J. C.** 1979. The beige mutation in the mouse. I. A stem cell predetermined impairment of natural killer cell function. J. Immunol. **123**:2168–2173.
44. **Roder, J. C., M. Lohmann-Matthes, W. Domzig, and H. Wigzell.** 1979. The beige mutation in the mouse. II. Selectivity of the natural killer (NK) cell defect. J. Immunol. **123**:2174–2181.
45. **Shand, F.** 1979. The immunopharmacology of cyclophosphamide. Int. J. Immunopharmacol. **1**:165–171.
46. **Shellam, G. R., J. E. Allen, J. M. Papadimitriou, and G. J. Bancroft.** 1981. Increased susceptibility to cytomegalovirus infection in beige mutant mice. Proc. Natl. Acad. Sci. USA **78**:5104–5109.
47. **Stein-Streilein, J., M. Bennett, D. Mann, and V. Kumar.** 1983. Natural killer cells in mouse lung: surface phenotype, target preference, and response to local influenza virus infection. J. Immunol. **131**:2699–2704.
48. **Swenson, F. J., and T. R. Kozel.** 1978. Phagocytosis of *Cryptococcus neoformans* by normal and thioglycolate-activated macrophages. Infect. Immun. **21**:714–720.
49. **Tacker, J. R., F. Farhi, and G. S. Bulmer.** 1972. Intracellular fate of *Cryptococcus neoformans*. Infect. Immun. **6**:162–167.
50. **Timonen, T., J. R. Ortaldo, and R. B. Herberman.** 1981. Characteristics of human large granular lymphocytes and relationship to natural killer cells and K cells. J. Exp. Med. **155**:569–582.
51. **Weindruch, R., B. H. Stevens, H. V. Raff, and R. L. Walford.** 1983. Influence of dietary restriction and aging on natural cell activity in mice. J. Immunol. **130**:993–996.
52. **Welsh, R. M.** 1981. Natural cell-mediated immunity during viral infection. Curr. Top. Microbiol. Immunol. **92**:83–106.
53. **Wiltrout, R. H., R. B. Herberman, S. Zhang, M. A. Chirigos, J. R. Ortaldo, K. M. Green, and J. E. Talmadge.** 1985. Role of organ-associated NK cells in decreased formation of experimental metastases in lung and liver. J. Immunol. **134**:4267–4275.
54. **Wiltrout, R. H., B. J. Mathieson, J. E. Talmadge, C. W. Reynolds, S. R. Zhang, R. B. Herberman, and J. R. Ortaldo.** 1984. Augmentation of organ associated natural killer activity by biological response modifiers. J. Exp. Med. **160**:1431–1449.