

AUGMENTATION OF ORGAN-ASSOCIATED NATURAL
KILLER ACTIVITY BY BIOLOGICAL RESPONSE MODIFIERS
Isolation and Characterization of Large Granular Lymphocytes from
the Liver

BY ROBERT H. WILTROUT,* BONNIE J. MATHIESON,†
JAMES E. TALMADGE,§ CRAIG W. REYNOLDS,† SHU-REN ZHANG,*
RONALD B. HERBERMAN,† AND JOHN R. ORTALDO‡

*From the *Laboratory of Molecular Immunoregulation, †Biological Therapeutics Branch, and
‡Preclinical Screening Laboratory, Program Resources Inc., and the Biological Response
Modifiers Program, National Cancer Institute, Frederick Cancer Research Facility,
Frederick, Maryland 21701*

Natural killer (NK)¹ activity has been implicated as an important immune mechanism in the prevention of experimental tumor metastasis in both the mouse (1–3) and the rat (4).² NK activity has also been suggested to be an important component of the immune response against virus-infected cells (5–7). In the human (8) and the rat (9), this NK activity has been closely associated with large granular lymphocytes (LGL). In both species, it has been possible to enrich for LGL, and concomitantly for NK activity, by fractionation of peripheral blood lymphocytes (PBL) on discontinuous gradients of Percoll. The ability to consistently obtain LGL in high purity has allowed studies of LGL to proceed steadily for both the rat (4, 10–12) and the human (13–15). However, the isolation of highly enriched LGL from the mouse has been more difficult (16–18). Generally, the LGL purity obtained by Percoll fractionation of mouse spleen cells has been only 20–40%, even when two sequential gradients have been employed (17). Higher purity of mouse LGL has been obtained from blood (17); however, studies of these cells have been limited by the relatively low yield of LGL. Similarly, LGL with NK activity have been isolated from the mucosa of the small intestine of mice (19, 20). These cells were morphologically and phenotypically

This project has been funded in part with Federal funds from the Department of Health and Human Services, under contract number NO1-CO-23910 with Program Resources, Inc. The contents of this publication do not necessarily reflect the views of policies of the Department of Health and Human Services nor does mention of trade names, commercial products, or organizations imply endorsement by the U. S. Government.

¹ *Abbreviations used in this paper:* asGM₁, asialo GM₁; BRM, biological response modifier; BSA, bovine serum albumin; FBS, fetal bovine serum; FCA, flow cytometry analysis; F1-GAMIg, fluoresceinated goat antibody to mouse Ig; F1-GARabIg, fluoresceinated goat anti-rabbit Ig; F1-GARatIg, fluoresceinated goat anti-rat Ig; HBSS, Hanks' balanced salt solution; IF, immunofluorescence; LAL, large agranular lymphocytes; LGL, large granular lymphocytes; LPS, lipopolysaccharide; MAb, monoclonal antibody; MVE-2, maleic anhydride divinyl ether; NC, natural cytotoxic; NK, natural killer; PBL, peripheral blood lymphocytes.

² Barlozzari, T., C. W. Reynolds, R. H. Wiltrott, and R. B. Herberman. Direct evidence for the role of NK cells in the inhibition of tumor metastasis. Manuscript submitted for publication.

similar to LGL obtained from spleen, except that the gut LGL were more readily depleted by treatment with anti-Thy-1.2⁺ antibody and complement than were splenic LGL.

Recently, we have linked increased resistance to metastasis formation in the lungs and livers of mice treated with the biological response modifier (BRM) maleic anhydride divinyl ether (MVE-2) to the presence of augmented NK activity localized in these organs.³ In the present paper, we describe the nature of the activated NK effector cells isolated from the liver. Specifically, we report the isolation, enrichment, surface phenotype, and cytolytic activity of activated LGL from perfused livers of mice treated with MVE-2 or heat-killed *Corynebacterium parvum*. The results demonstrate that large numbers of highly purified LGL can be obtained from the livers of BRM-treated mice. Further, the results suggest that at least some BRMs may induce antiviral or antimetastatic therapeutic effects through augmentation of organ-associated natural immunity.

Materials and Methods

Animals. Inbred male and female C57BL/6N mice were obtained from the Animal Production Area, National Cancer Institute-Frederick Cancer Research Facility, Frederick, MD, and were routinely used at 7-10 wk of age.

Tumor Cell Lines. Tumor cells used as targets in these studies were the YAC-1 lymphoma of A/Sn origin (21), the L5178Y lymphoma of DBA/2 mice (22), the P815 mastocytoma of DBA/2 mice (23), the MBL-2 lymphoma of C57BL/6 mice (24), the WEHI-164 fibrosarcoma of BALB/c mice (25), and Meth A, a chemically induced fibrosarcoma of BALB/c mice (26). The cells were maintained in continuous *in vitro* culture in RPMI 1640 (M. A. Bioproducts, Walkersville, MD) supplemented with 10% fetal bovine serum (FBS) (Sterile Systems, Logan, UT), 100 U/ml of penicillin and 100 µg streptomycin (P/S), 50 µg/ml gentamycin (Schering Corp., Kenilworth, NJ), and 300 µg/ml L-glutamine (complete medium). The RPMI 1640 and FBS batches were demonstrated by *Limulus* amoebocyte test (M. A. Bioproducts) to contain <0.1 ng/ml of lipopolysaccharide (LPS). All tumor cell lines have been shown to be free of mycoplasma (Flow Laboratories, McLean, VA), as well as the following viruses: Sendai, pneumonia virus of mice, minute virus of mice, lymphocytic choriomeningitis virus, polyoma virus, mouse hepatitis virus, mouse adenovirus, Ectromelia virus, and lactic dehydrogenase elevating virus (Animal Health Diagnostic Laboratory, National Cancer Institute-Frederick Cancer Research Facility).

Radioisotopic Labeling Procedures. ⁵¹Cr was obtained from New England Nuclear (Boston, MA) at a concentration of 1 mCi/ml. Labeling with ⁵¹Cr was performed by incubating 5 × 10⁶ tumor cells in 0.5 ml complete medium at 37°C with 100 µCi ⁵¹Cr for 1 h. Labeled cells were then washed two times in 50 ml complete medium and resuspended to the proper concentration.

Biological Response Modifiers. NK activity was augmented by the intravenous or intraperitoneal injection of 25 mg/kg of the pyran copolymer MVE-2, or by the intraperitoneal injection of 25 mg/kg of the pyran copolymer MVE-2, by the intraperitoneal injection of 35 mg/kg formalin-killed *C. parvum* (strain CN 6134; Burroughs Wellcome, Inc., Research Triangle Park, NC). Both MVE-2 (27) and *C. parvum* were injected 1-12 d before harvest, depending on the nature of various experiments.

Anti-Asialo GM₁ Serum. Rabbit antiserum to asialo GM₁ (asGM₁) was obtained from Wako Pure Chemical Co., Dallas, TX. The specificity of such antisera for asGM₁ has been previously documented (28). The standard regimen of NK suppression *in vivo* has been

³ Wiltrout, R. H., R. B. Herberman, M. A. Chirigos, J. R. Ortaldo, K. M. Green, Jr., and J. E. Talmadge. Role of NK cells in lung and liver in decreased formation of experimental metastases. Manuscript submitted for publication.

determined such that 0.2 ml of a 1:20 dilution of anti-asGM₁ serum eliminates >80% of NK activity in blood and spleen. Optimal suppression of NK activity occurs when the antisera are administered 1–3 d before assay.

Isolation of Effector Cells from the Liver. Cells were isolated from the liver using the method of Richman et al. (29), with some modifications. Mice were killed by cervical dislocation; the peritoneal cavity was aseptically exposed and the inferior vena cava was cut to allow rapid exsanguination. Next, the hepatic portal vein was exposed and catheterized with a 21-gauge butterfly needle, and 8–10 ml prewarmed RPMI 1640 medium with 5% fetal bovine serum (FBS) was slowly injected to flush blood from the hepatic vasculature. When properly performed, the perfused liver was completely blanched. Any livers or lobes that were not completely blanched were discarded. The liver was then excised and the gallbladder was removed. Livers were then extensively minced into small pieces with surgical scissors, gently forced through 50-gauge stainless steel mesh using a sterile syringe plunger, and placed at 4°C in a 50-ml polypropylene tube. This extract was centrifuged at 600 *g* for 10 min, and the supernatant was removed. 10 ml of prewarmed enzyme solution, containing 0.05% collagenase type II (Sigma Chemical Co., St. Louis, MO) and 500 U/ml DNAase type I (Sigma Chemical Co.) in Hanks' balanced salt solution (HBSS) plus 5% FBS, was added per milliliter of packed liver extract. The enzyme extract mixture was incubated in a 37°C water bath for 8–10 min with constant agitation, and the enzymatic digest washed two times in cold HBSS (without Ca⁺⁺ or Mg⁺⁺). When properly performed, this procedure yields a cell pellet that contains few erythrocytes, which indicates that the isolated cells are not substantially contaminated by lymphocytes from the peripheral blood. The enzymatically digested liver was then resuspended in a minimal amount of cold HBSS and 30% metrizamide in Gey's solution at a final ratio of 7 parts metrizamide per 5 parts packed liver digest. 3–5 ml of the mixture was transferred to 15-ml conical tubes and overlaid with 1.5 ml PBS. This one-step metrizamide gradient was then centrifuged at 1,400 *g* for 20 min at 4°C, the nonparenchymal cell layer was carefully removed from the metrizamide PBS interface with a pasteur pipette, and the cells were washed two times in RPMI 1640 plus 5% FBS. The composition of the nonparenchymal cells was then ascertained by Wright-Giemsa stain of a cytocentrifuge preparation (>95% leukocytes), and the cells were resuspended to the desired cell concentration in RPMI 1640 supplemented with 5% FBS, 20 mM Hepes buffer (M. A. Bioproducts), 1 mM sodium pyruvate (Gibco Laboratories, Grand Island, NY), P/S, and L-glutamine (assay medium).

Isolation of LGL. LGL were obtained after the metrizamide purification of mononuclear cells from suspensions of enzymatically digested liver by a modification of separation procedures previously described for human LGL (8, 13, 14). Briefly, nonadherent lymphocytes were obtained by removal of adherent cells on nylon wool columns (37°C for 45 min) and the eluted cells (20–100 × 10⁶ cells/gradient) were then fractionated by centrifugation at 300 *g* for 30 min, on a seven-step discontinuous density gradient (osmolality, 290 mosmol) of Percoll (Pharmacia Fine Chemicals, Uppsala, Sweden) at concentrations of 38.6, 47.6, 52.1, 56.6, 61.1, 65.6, and 70.1%. LGL were collected from the uppermost low-density fractions (fractions 1 and 2), while T cells were collected from the high-density bottom fractions (fractions 4–6). Cell preparations were evaluated for morphology by microscopic analysis of Giemsa-stained cytocentrifuge preparations.

Assay for NK Cell-mediated Cytotoxicity. Various numbers of spleen, liver, or peritoneal effector cells were mixed in quadruplicate with 5 × 10⁵ ⁵¹Cr-labeled target cells in 96-well, round-bottomed microtiter plates (Linbro Scientific Co., Hamden, CT) in a total volume of 0.2 ml assay medium. Plates were incubated for 4 h, supernatants were removed from wells by the Titertek automatic harvesting system (Flow Laboratories, McLean, VA), and the radioactivity was counted in a gamma counter (Beckman Instruments, Inc., Fullerton, CA). Control cultures consisted of ⁵¹Cr-labeled target cells incubated in assay medium alone.

The percentage specific release of ⁵¹Cr was calculated by the following formula: Percent specific cytotoxicity = [(mean cpm released from test well – mean cpm released from target

cells alone)/(mean total cpm)] \times 100. Results are also expressed as lytic units (LU)/ 10^7 cells, with LU being the number of effector cells required to lyse 30% of the target cells.

Immunofluorescence Experiments. Immunofluorescence (IF) of LGL and T cell surface antigens was detected by primary incubation with monoclonal antibodies (MAb), whenever available followed by a secondary incubation with a fluoresceinated secondary antibody. LGL and T cell-enriched suspensions obtained from Percoll fractions (14) were adjusted to 2×10^7 cells/ml and 50 μ l of cells (1×10^6 cells) were incubated with 10–50 μ l MAb, depending on the source and concentration. Cells plus antibody were incubated for 45 min and then washed two times in a 2-ml vol of HBSS with bovine serum albumin (BSA) and NaN_3 . A second incubation with an appropriately titered fluoresceinated reagent was also done for 45 min. After incubation with the secondary antibody, the cells were again washed twice and suspended in 0.4 ml HBSS plus BSA plus NaN_3 for flow cytometry analysis (FCA). For some MAb, isotype-specific developing reagents were used; however, in most cases, fluoresceinated, affinity-purified goat antibody to mouse Ig (Fl-GAMIg), goat anti-rabbit Ig (Fl-GARabIg), or goat anti-rat Ig (Fl-GARatIg) were required for the secondary developing reagent. Fl-GARabIg and Fl-GARatIg were obtained as F(ab')₂ fragments from Cappel Laboratories (Cochranville, PA). The other reagents have been described elsewhere (30) and were obtained from Dr. B. J. Fowlkes (Laboratory of Microbial Immunity, National Institute of Allergy and Infectious Diseases, Bethesda, MD). The MAb reagents that were used, along with the source of the reagents and the pertinent references for these reagents, are indicated in the figure legends.

FCA. Immunofluorescence was analyzed on a Cytofluorograf System 30-H with a 2150 computer (Ortho Diagnostic Systems Inc., Westwood, MA) that was modified to provide simultaneous measurement of forward and right angle light scatter (488 nm) and red (>600 nm) and green (530 nm) fluorescence. Cells were illuminated by a 4-W argon laser (Lexel Corp., Palo Alto, CA) emitting 500 mW of 488-nm light. Cells analyzed for IF were selected by forward light scatter, and dead cells, identified by the uptake of propidium iodide (red fluorescence), were excluded from green fluorescence analysis. A green fluorescence histogram of 1,000 channel resolution was collected from 20,000 viable cells for each sample analyzed. The percentage of positive cells was calculated by integration of the experimental and control (secondary fluoresceinated reagent alone) histogram.

Statistics. Statistical analyses were performed by Student's *t* test.

Results

Augmentation of NK Activity in Spleen and Liver after Administration of MVE-2. Because our previous observations demonstrated a relationship between organ-associated NK activity in MVE-2-treated mice and resistance to metastasis,³ and since MVE-2 augments NK activity in the spleen (31), we were interested in determining whether NK activity in other nonlymphoid organs was also augmented by MVE-2 treatment. The experiment shown in Fig. 1 demonstrated that as in the spleen, liver NK activity peaked at 3 d after the intravenous injection of MVE-2. However, the NK activity from the liver remained significantly ($P < 0.05$ to < 0.005) elevated through at least day 11, and, at the same effector-to-target ratios, was always higher than the activity seen with unfractionated spleen cells. The relationship in NK activity among unfractionated cells isolated from the liver at various times after MVE-2 treatment is better shown in Fig. 2. At 3 d after MVE-2 injection, high levels of specific cytotoxicity against the YAC-1 target cells (>40%) were observed even at effector-to-target ratios as low as 12:1. These results are particularly impressive when one considers that this level of hepatic NK activity was observed with unfractionated leukocyte populations from the liver.

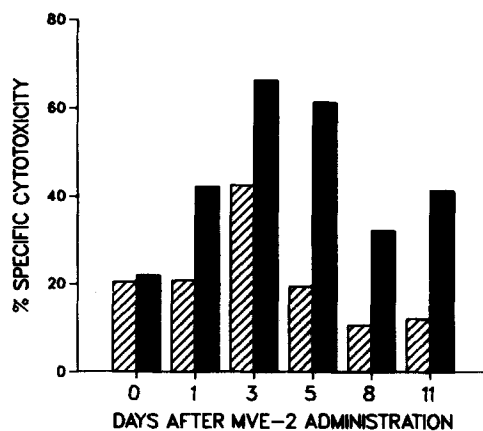


FIGURE 1. Augmentation of NK activity in spleen and liver following administration of MVE-2. Cells from spleen (▨) and liver (■) were obtained as described in the Materials and Methods from normal C57BL/6 mice or mice injected intravenously 1–11 d earlier with MVE-2. These cells were tested against ^{51}Cr -labeled YAC-1 tumor cells for 4 h for assessment of NK activity. The effector-to-target ratio was 100:1. Splenic NK was significantly augmented ($P < 0.01$) only on day 3, while hepatic NK was significantly augmented ($P < 0.005$ – 0.05) at all times tested.

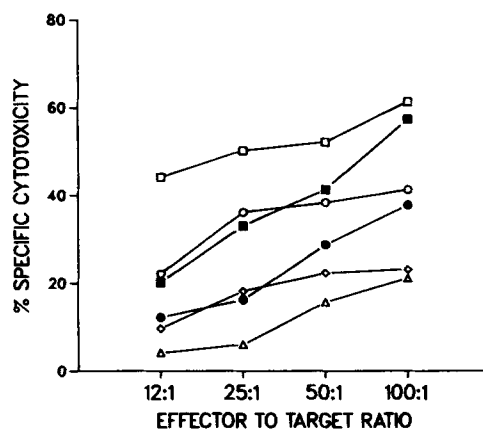


FIGURE 2. Augmentation of hepatic NK activity by administration of MVE-2. MVE-2 was administered to C57BL/6 mice intravenously 1–11 before harvest of the livers for NK assessment. Leukocytes isolated from the livers were reacted at various effector-to-target ratios of ^{51}Cr -labeled YAC-1 cells. Δ, normal; ○, 1 d; □, 3d; ■, 5 d; ◇, 8 d; ●, 11 d.

Augmentation of Peritoneal and Liver NK Activity by Administration of C. parvum. Because MVE-2 increased resistance to metastasis³ and strongly augmented levels of NK activity in the liver (Figs. 1 and 2), it was of interest to determine whether other antimetastatic BRMs would also augment liver-associated NK activity. The results shown in Fig. 3 demonstrate that after *C. parvum* administration, NK activity of leukocytes isolated from the liver reached peak levels on day 3 and declined to normal levels by day 10. Significant augmentation of cytotoxicity ($P < 0.01$ to < 0.05) was observed between days 1 and 8. Peritoneal NK activity, similar to the liver-associated NK activity, peaked 3–6 d after *C.*

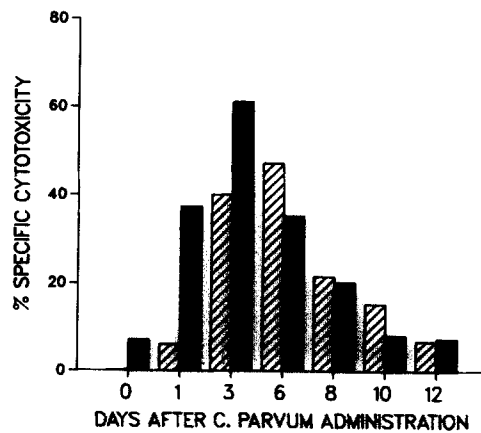


FIGURE 3. Augmentation of peritoneal and liver NK activity by administration of *C. parvum*. *C. parvum* was administered intraperitoneally to C57BL/6 mice 1–12 d before the harvest of peritoneal exudate or liver-derived cells. These cell populations were then incubated with ^{51}Cr -labeled YAC-1 for 4 h and levels of NK activity were assessed at an effector-to-target ratio of 50:1. Similar results were obtained at other ratios. Both peritoneal and hepatic NK was significantly augmented at 1, 3, and 6 d ($P < 0.01$), as well as 8 d ($P < 0.05$). ▨, PEC; ■, liver.

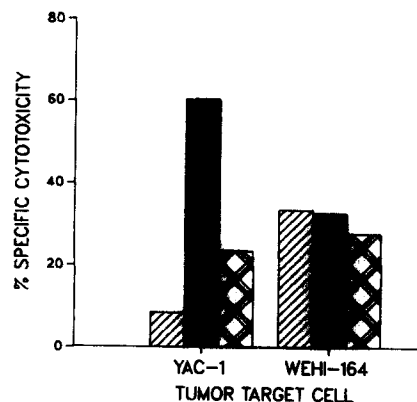


FIGURE 4. Effects of MVE-2 and anti-asGM₁ serum administration on NK and NC activities in the liver. Liver-derived mononuclear cells were isolated from normal mice (▨), mice treated 3 d earlier with MVE-2 and 1 d earlier with anti-asGM₁ serum (cross-hatched bar) or with just MVE-2 three days earlier (■). These cells were then tested against with ^{51}Cr -labeled YAC-1 for 4 h (NK) or with ^{51}Cr -labeled WEHI-164 for 18 h (NC). The effector-to-target ratio was 50:1 for both NK and NC. Lysis of YAC-1 was significantly augmented by MVE-2 ($P < 0.01$) and the MVE-2 augmentation was significantly reduced by anti-asGM₁ serum treatment ($P < 0.01$).

parvum administration and then rapidly declined. The results indicate that although both MVE-2 and *C. parvum* augment NK activity in the liver, the effect is more transient for *C. parvum*.

Sensitivity of Augmented Liver NK Activity to Anti-asGM₁ Serum. Spleen and blood NK activity has been previously shown (3, 28, 32, 33) to be sensitive to in vitro or in vivo treatment with anti-asGM₁ serum. This depression of NK activity by anti-asGM₁ serum has also been correlated with an increase in metastasis (3).

The study shown in Fig. 4 was designed to determine whether the cells mediating NK activity from the livers of MVE-2-treated mice also expressed the NK-associated marker asGM₁. In this experiment, the MVE-2-augmented NK activity of liver-associated cells was reduced ~65% when anti-asGM₁ serum was administered 1 d before assay. These results demonstrate that the augmented NK activity induced by MVE-2 in the liver could be significantly ($P < 0.01$) depleted by treatment in vivo with anti-asGM₁ serum.

Since natural cytotoxic (NC) cells have also been implicated as antitumor effectors (26), we tested for the presence of NC activity in liver, and its modulation by MVE-2 and anti-asGM₁ serum. Interestingly, the nonparenchymal cells obtained from normal liver tissue had considerable NC activity as assessed by lysis of WEHI-164 cells (Fig. 4). However, in contrast to NK activity, the NC activity from the liver was neither augmented by MVE-2 nor reduced by treatment with anti-asGM₁ serum (Fig. 4).

Isolation and Enrichment of LGL from the Livers of MVE-2-treated Mice. The findings of augmented NK activity after BRM treatment led us to postulate that the liver would contain LGL, which have been characterized morphologically as the cells mediating NK activity (4, 8–13, 16–20). Further, we postulated that since the NK activity of MVE-2-treated mice was higher in leukocytes isolated from the liver than in spleen or peritoneal cell isolates, the liver-derived cell population might contain a high percentage of LGL that could be used for further enrichment of LGL. To test these postulates, we obtained leukocyte-rich preparations by metrizamide gradient fractionation of enzymatically dissociated livers from normal mice and from mice treated earlier with BRMs. Routinely, $\sim 0.5\text{--}2.0 \times 10^6$ total nonparenchymal cells were recovered per normal mouse, 25–40% of which were macrophages (Kupffer cells, as characterized by phagocytosis and esterase positivity) and 45–60% lymphocytes. After the administration of BRMs, the total number of cells recovered per mouse increased markedly to $5\text{--}10 \times 10^6$ for MVE-2 and $5\text{--}20 \times 10^6$ for *C. parvum*, depending on the time of BRM administration. In spite of this increase in total cell number, the percentages of macrophages and lymphocytes obtained from the livers of BRM-treated mice remained similar to those observed for normal control mice.

Morphological characterization of the metrizamide gradient-purified leukocytes revealed that the percent LGL increased from 3–8% in normal mice to 10–15% in BRM-treated mice. The total number of hepatic LGL recovered from BRM-treated mice was consistently in the range of $2.0\text{--}2.5 \times 10^6$ per mouse compared with $0.05\text{--}0.2 \times 10^6$ per normal mouse, a 10–50-fold increase (Table I). This increase in the number of LGL isolated from perfused liver (i.e., nonperipheral blood cells) was accompanied by an appreciable increase in the number of mononuclear cells observed in the tissues by histological examination of liver sections (Wiltrout, R. H., and C. W. Reynolds, manuscript in preparation). These cells were observed initially as perivascular cuffing and later were uniformly distributed throughout the liver parenchyma.

To further enrich for liver-derived LGL, metrizamide-purified leukocytes were depleted of adherent cells by passage over nylon wool columns. The resulting suspension obtained from normal livers contained an average of 10% LGL, while the nylon wool-passed leukocyte preparations from MVE-2-treated

TABLE I
Total Number of LGL Obtained from the Livers of Normal and
BRM-treated Mice*

Treatment	Total LGL $\times 10^{-6}$ /liver	n
None	0.05-0.2	10
MVE-2	2.0-2.5	8
<i>C. parvum</i>	2.0-2.5	6

* Determined by enumeration of LGL in metrizamide gradient-purified leukocyte preparations obtained from enzymatically digested livers. The number of experiments (n) used to obtain the pooled data is shown for each treatment.

TABLE II
Enrichment for Liver LGL and NK Activity by Depletion of Adherent Cells and Fractionation
on Percoll Density Gradients

Treatment in vivo*	Enrichment in vitro		Percent total cells that were:			Lytic units (30%) [§]	Total LGL recovered per liver [†]
	NW [‡]	Percoll	LGL	LAL	LGL + LAL		
None	+	—	10	13	23	3	5.5×10^4
MVE-2	+	—	36	26	62	546	1.1×10^6
	+	F ₁	69	22	91	1,829	3.4×10^5
	+	F ₂	49	26	75	521	2.0×10^5
	+	F ₃	10	10	20	13	4.0×10^4
	+	F ₄	5	5	10	0	7.1×10^3
MVE-2 + Anti-asGM ₁	+	—	18	27	45	215	1.1×10^5

* C57BL/6 mice were injected intravenously with 25 mg/kg MVE-2 three days before harvest. Some mice also received 0.2 ml of a 1:15 dilution of anti-asGM₁ serum 1 d before harvest.

[‡] NW, nylon wool-nonadherent cells with adherent cells removed by incubation on nylon wool columns as described in Materials and Methods.

[§] Percent specific release was assessed against YAC-1 and lytic units calculated per 1×10^7 cells.

[†] Following nylon wool with or without Percoll gradient separation.

mice were enriched in LGL to 36% (Table II). Most of the non-LGL cells obtained from MVE-2-treated mice following nylon wool passage were large agranular lymphocytes (LAL). LAL are morphologically similar to LGL, but possess no visible azurophilic cytoplasmic granules and may be related to LGL (17, 34). As with the LGL, the percent of these cells was consistently increased twofold over normal mice (Table II). Similar results were obtained when *C. parvum* was used as the stimulus for augmenting hepatic NK activity.

Increases in the percentage of LGL isolatable from the lungs, in addition to the liver, were also observed after MVE-2 treatment (data not shown).

Since the increase in number and percentage of liver-associated LGL in MVE-2-treated mice correlated well with the augmented NK activity (Table II), further enrichment of LGL by fractionation on discontinuous Percoll density gradients was performed. This fractionation resulted in even higher levels of both LGL and NK activity, with the cells from fraction 1 enriched to 69% LGL and those from fraction 2 to 49% of LGL. Cells from these two fractions accounted for virtually all of the NK activity seen. In contrast, fractions 3 and 4 were mostly

small lymphocytes, and had very low NK activity. Consistent with the marked reduction in NK activity, treatment with anti-asGM₁ serum *in vivo* reduced both the percentage (from 36 to 18%) and total number of LGL (by 90%) isolated from the liver (Table II).

Target Specificity of LGL Isolated from Livers of MVE-2-treated Mice. Selectivity of target cell lysis has been one of the classic characteristics used for the definition of NK activity and permits its discrimination from macrophage-mediated cytotoxicity (35, 36). To determine whether liver LGL mediated tumor lysis with the target selectivity expected for NK cells, we tested Percoll-enriched LGL from the livers of mice treated with MVE-2 against a panel of NK-sensitive and NK-resistant tumor cell lines (Fig. 5). Liver-derived LGL efficiently lysed YAC-1 in a dose-dependent manner, even at effector-to-target ratios as low as 0.7:1. Conversely, three NK-resistant tumor targets (P815, L5178Y, and MBL-2) were poorly lysed even at the highest effector cell concentration used, in spite of the fact that all are good targets for macrophages and cytotoxic T cells (31). These studies demonstrate that liver-derived LGL from MVE-treated mice possess the cytotoxic specificity commonly attributed to NK cells, but not to cytotoxic T cells or macrophages.

Surface Phenotype of LGL Isolated from the Liver. To further characterize the highly lytic LGL obtained from the livers of mice treated with MVE-2 or *C. parvum*, and to compare the surface phenotype of liver-derived LGL with that reported for other LGL populations, Percoll-enriched LGL were analyzed for expression of cell surface markers by FCA. Similar results were obtained with cells from mice treated with both BRMs (Table III). Examples of the FCA are shown in Fig. 6. Percoll-enriched, liver-derived LGL expressed Ly-5 and asGM₁, antigens previously shown to be associated with murine NK cells (28, 32, 33, 37-41), in high amounts. Similarly, the fraction 1 liver LGL were positive for Qa-5 expression, in agreement with previous reports on the mouse NK cell phenotype (39-41). However, in contrast to the low Thy-1.2 phenotype previ-

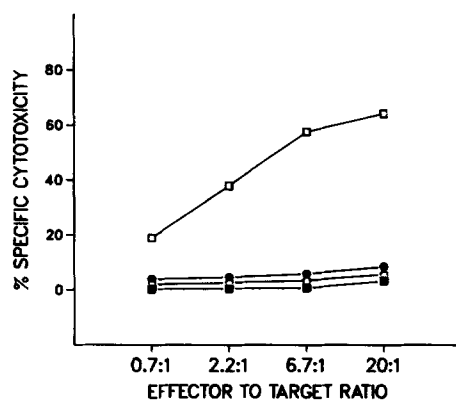


FIGURE 5. Target specificity of LGL isolated from livers of MVE-2-treated mice. Mice were injected intravenously with MVE-2 3 d before harvest and hepatic LGL were isolated as described in Materials and Methods. The liver LGL from Percoll fraction 1 (69% LGL) were then tested against ⁵¹Cr-labeled YAC-1 (□), P815 (■), L5178Y (○), or MBL-2 (●) for 4 h for assessment of cytotoxicity.

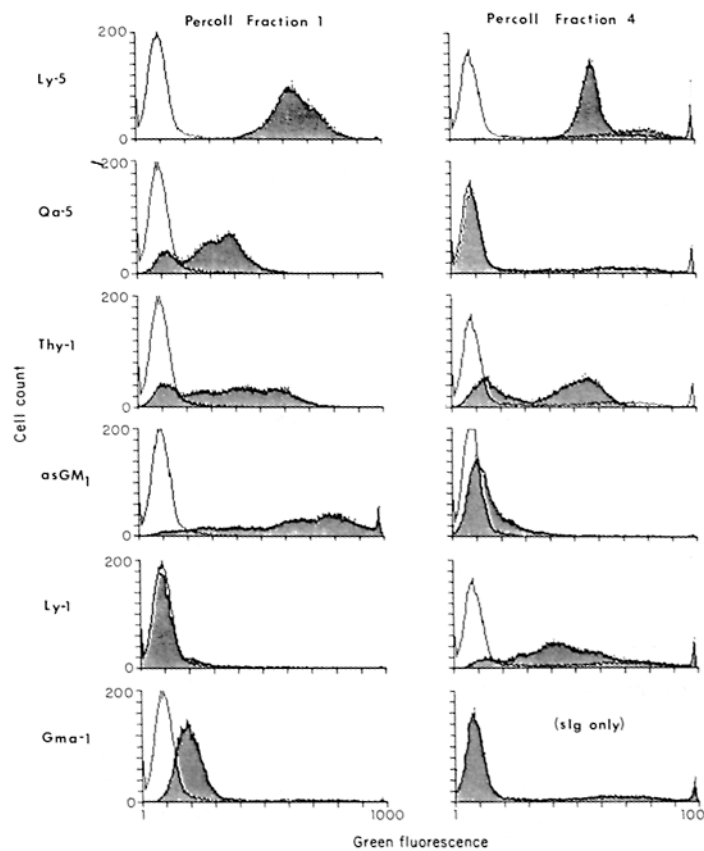


FIGURE 6. Flow cytometry IF histograms of cell surface antigens. Percoll fractions of cells isolated from the liver of MVE-2-treated mice, enriched for LGL (fraction 1) or small lymphocytes (fraction 4), were treated with MAb or rabbit antibody plus appropriate secondary fluorescent reagents. The reagents used were as follows: anti-Ly-5.1, (New England Nuclear), 10 μ l of 1:400-diluted ascites; anti-Qa5, clone B16-167 obtained from Dr. Ulrich Hammerling (Sloan-Kettering Institute, New York), 50 μ l of culture supernatant; directly fluoresceinated anti-Thy-1.2 (Becton-Dickinson, Palo Alto, CA), 50 μ l of 1:100-diluted stock; anti-Ly-1.2 (New England Nuclear), 10 μ l of 1:200-diluted ascites; anti-Gma-1.2 hybridoma ascites obtained from Dr. Fung-Win Shen (Sloan-Kettering Institute), 10 μ l of 1:200-diluted ascites. Cell samples treated with MAb to Ly-5, Qa-5, Ly-1, and Gma-1 were subsequently treated with a polyvalent F1-GAM1g that also detects slg. The background in fraction 1 with this reagent was 5.3%, whereas there were 29% slg⁺ cells in fraction 4 (seen in the lower right panel). When anti-asGM₁ was used, F1-GARab1g was used as the secondary reagent. The appropriate control histograms are indicated by the unfilled histograms in each panel. Cells in fraction 1 were 99.8% Ly-5⁺, 77% Qa-5⁺, 75% Thy-1⁺, 93% asGM₁⁺, 5% (net) Lyt-1⁺, and 97% Gma-1⁺ (with greater IF than the median control value). By comparison, cells in fraction 4 were 98.9% Ly-5⁺ (including 29% slg⁺), 4% (net) Qa-5⁺, 61.5% Thy-1⁺ (greater than channel 300), 35.8% asGM₁⁺ (greater than channel 150), and 60.3% Lyt-1⁺ (net value with 29% slg⁺ cells excluded).

ously reported for NK cells from spleen or blood (26, 41-43), the liver-derived LGL were strongly Thy-1⁺, regardless of which NK-augmenting agent was used. The majority of liver-derived LGL were also positive for Gma-1.2, a myelomonocytic antigen (44), as well as for the Mac-1 (45) and/or M57 (46) markers (Table III). The enriched LGL population were also routinely weakly positive for Fc

TABLE III
Antigenic Phenotype of LGL Isolated from Mouse Liver

Cell surface markers	Treatment in vivo	
	MVE-2	<i>C. parvum</i>
Ly-1	—	—
Lyt-2	—	—
L3T4	—	ND*
Ly-5	+	+
Thy-1	+	+
asGM ₁	+	ND
Qa-5	+	+
Gma-1	+	ND
Mac-1/M57	+	ND
sIg	—	—

* ND, not determined.

receptors and negative for surface Ig (sIg⁻). A summary of these results is shown in Table III. Regardless of the augmenting agent that was used, the highly enriched fraction 1 hepatic LGL were devoid of several specific T cell markers such as Ly-1, Lyt-2, and L3T4 (47) (Table III). The presence of these markers might have indicated in vivo expansion or induction of a nonspecific cytolytic T cell population similar to the in vitro interleukin 2-expanded cells described by others (48, 49). Conversely, the T cell-enriched fraction 4 is strongly positive for Ly-1, exhibits only low amounts of asGM₁, and is negative for Qa-5 and Gma-1 (Fig. 6).

Discussion

This paper describes the isolation and characterization of large numbers of NK-active cells from the livers of mice treated with the BRMs MVE-2 and *C. parvum*. These results are of particular interest since vital organs, including the liver, are often sites of tumor metastasis formation. We have previously postulated³ that the normally occurring or BRM-induced NK activity within these nonlymphoid organs may serve as an important component of the natural resistance to metastasis formation. In support of this postulate, we have correlated the induction of augmented organ-associated NK activity with immunoprophylactic resistance to the formation of experimental metastases, which further suggests the importance of these cells in organ-associated antitumor immune defense mechanisms.

The present studies have demonstrated that cells isolated from the livers of normal mice had low NK activity, while administration of MVE-2 or *C. parvum* dramatically augmented NK activity in the cells isolated from the liver (Figs. 1–3). It should be emphasized that the majority of these cells isolated from the liver are not circulating PBL, but represent a population consisting of some cells strongly adherent to the vascular endothelium (not removed by extensive perfusion), and many cells already extravasated into the liver interstitium. The evidence that argues against a significant contamination by PBL includes a lack of erythrocyte contamination in the enzymatically digested liver preparations.

Additionally, the augmentation of NK activity in blood is much smaller than that seen with leukocytes from liver preparations.³ Furthermore, these increased levels of liver-associated NK activity correlated directly with the adherence of mononuclear cells to the microvasculature of both lung and liver with subsequent pronounced mononuclear cell infiltration into the liver tissue, detectable by histological examination (Wiltrout, R. H., and C. W. Reynolds, manuscript in preparation). These results are consistent with the large increase in the total number of cells isolated from the livers of BRM-treated mice, and strongly suggest that many of these cells are liver-associated NK cells involved in the organ-associated immune defenses.

Although our previous results had demonstrated the presence of NK-active lymphocytes in nonlymphoid organs, and associated their presence with the therapeutic effects of MVE-2, the nature of the liver-associated NK-active cells remained unclear. Therefore, to address this issue, we characterized the specificity of these BRM-induced, liver-associated cytolytic cells. The cytotoxic selectivity of these liver-derived cells was characteristic for NK activity (35, 36), with efficient lysis of YAC-1 but not of NK-resistant tumor cells in 4 h (Fig. 5). This cytotoxicity was greatly reduced by a single administration of the NK-suppressive anti-asGM₁ serum (Fig. 4) and could be completely eliminated by a double-treatment regimen (data not shown). In contrast, normal mice had moderate levels of NC activity, which were neither augmented by MVE-2 nor reduced by treatment with anti-asGM₁ serum (Fig. 4). Therefore, MVE-2 induced an augmentation of NK but not NC activity.

Since NK activity in lymphoid tissue has been associated with LGL (8, 9, 16–18), we performed studies to determine if the presence of NK-active cells from perfused livers of MVE-2-treated mice might be associated with LGL. These studies revealed that some of the increase in mononuclear cells in the livers of MVE-2-treated mice was at least partially due to LGL (Tables I and II) and that the enrichment for LGL (fraction 1) correlated with greatly increased NK activity (Table II). Further evidence for a role of LGL in liver NK activity came from experiments with anti-asGM₁ serum treatment *in vivo*, which decreased NK activity by >60% and the total number of LGL present in livers from MVE-2-treated mice (Table II) by ~90%.

It should be noted that treatment with MVE-2 did not increase liver-associated NC activity on a per cell basis (Fig. 4). However, there was an appreciable increase in the total number of cells isolated from the livers of MVE-2-treated mice. Therefore, there was also an increase in the total NC per liver following MVE-2 administration. In addition, although anti-asGM₁ treatment decreased NK activity, it did not decrease NC activity. This result demonstrates that although MVE-2 induced an increase in the total NC activity per liver, these NC cells were not sensitive to treatment with anti-asGM₁ serum. The lack of effect by anti-asGM₁ in reducing NC activity confirms a previous observation by Lattime et al. (50), and extends these findings to tissue-associated NC activity, which indicates that anti-asGM₁ serum may be a useful reagent for further delineating the cell types responsible for NK or NC activities and their relative antimetastatic capabilities *in vivo*.

Although several cell surface markers such as Ly-5 (T-200), Qa-5, and asGM₁

(32, 37, 38, 40, 42, 51) have been reported to be present on NK cells in the mouse, the phenotypic characterization of mouse LGL has proceeded more slowly than has the study of rat or human LGL. In fact, most of the NK phenotype data to date have depended on selective cytolytic elimination (41, 42, 43, 52) or positive selection techniques that have been capable of selecting for only one marker at a time (37, 53). Perhaps the greatest limitation in the characterization of the cell surface phenotype of mouse NK cells has been the difficulty obtaining highly purified cell populations in sufficient numbers to perform careful phenotypic analyses. Attempts to obtain mouse LGL by density sedimentation in BSA or Percoll have yielded low numbers of cells and/or only partially enriched LGL populations. In contrast, the ability to obtain large numbers of highly purified LGL from the livers of mice treated with MVE-2 has allowed us to better characterize these mouse LGL.

The surface phenotype of LGL obtained from the livers of MVE-2-treated mice was asGM⁺, Thy-1⁺, Mac-1⁺, weakly Qa-5⁺ and Gma-1⁺, and Ly-1⁻, Lyt-2⁻, L3T4⁻, and sIg⁻. With one exception, the liver LGL were quite similar in expression of surface markers to NK-active cells isolated from blood and spleen. However, the liver-derived LGL were brightly Thy-1⁺ as assessed by FCA (Table III, Fig. 6), whereas previous data by cytolysis (26, 41, 42, 43, 50, 52) and our own studies by FCA (Mathieson, B. J., R. Winkler, W. R. Overton, and L. Mason, manuscript in preparation) would indicate that blood and spleen NK cells or LGL express much lower amounts of Thy-1.

Finally, macrophage/myelomonocytic antigens have been recognized on human peripheral blood LGL (14) and associated with mouse NK activity (45, 46), but their expression on LGL from other nonlymphoid organs has not been examined. Thus, our findings that the liver LGL express these antigens may reflect further heterogeneity associated with either the activation state or the lineage(s) of LGL.

In contrast to peripheral blood or spleen NK cells, much less is known about the phenotype of other tissue-associated LGL. In a small number of experiments, NK-active cells have been isolated from the lungs (54) and small intestine (19), and these cells have been characterized for cytotoxic function, cell surface phenotype, and morphology. Stein-Streilein et al. (54) have demonstrated the presence of NK-active cells in the lungs of mice infected intratracheally with influenza virus, as well as a local augmentation of NK activity in the lung. The cells mediating this virus-augmented NK activity expressed asGM₁ and Ly-5, as did our highly active LGL isolated from the livers of BRM-treated mice. However, the liver LGL were strongly Thy-1⁺, while lung NK-active cells were reported to be Thy-1⁻ (54). By contrast, gut-associated LGL, with high NK activity, have been reported to be Thy-1⁺, but asGM₁ negative (19). Therefore, the LGL population isolated from the liver following MVE-2 or *C. parvum* treatment appears functionally similar in terms of target cell specificity (Figs. 2 and 5), but phenotypically distinct from other tissue-associated sources of NK-active cells.

Overall, the findings that NK-active cells from spleen, blood, lung, gut, and liver are phenotypically similar, but not identical, further emphasize the possible heterogeneity in NK populations derived from various tissues. This heterogeneity

may reflect different maturational states of NK cells or, alternatively, different NK phenotypes may be determined by the activation state of a single lineage. Different stimuli, such as viruses (in the lung [54]) or MVE-2 (in lung and liver) may induce LGL to achieve different activation stages, which result in altered phenotype. Alternatively, the results may indicate stable heterogeneity of NK-active cell subsets or lineage in various anatomical compartments. At present, the data to distinguish between these alternatives are not available.

The basis for the greatly augmented NK activity and LGL number in the livers of mice treated with MVE-2 or *C. parvum* is not completely resolved. There are three obvious possibilities for this augmentation of hepatic NK activity. First, we have studied the effects of the enzyme cocktail, used to dissociate the tissues, on the NK activity of normal spleen cells. Like Stein-Streilein et al. (54), we have observed no augmentation of NK activity following enzyme treatment, and therefore conclude that the increased NK activity associated with the liver was not a result of the enzyme treatment augmenting pre-existent NK activity. In addition, the large increase in LGL number could not possibly be attributed to the enzyme treatment. The second possible explanation for an increase in NK activity in the liver is that there was some NK activity present in the livers of normal mice, and that the administration of certain BRMs may induce the proliferation and activation of a pre-existent liver LGL pool. The proliferation of NK cells in response to virus infections has previously been reported (55), and we have preliminary data which would suggest that the number of cells in G₁-S phase is increased in MVE-2-treated mice over what might be expected in normal mice (Wiltrott, R. H., and J. R. Ortaldo, unpublished observation). However, the rate of proliferation induced by MVE-2 appears to be insufficient to account for the rapid increase in the number of LGL and NK activity. The third possibility for the increase in liver NK activity following BRM treatment would be a rapid influx of LGL from the blood. To address this possibility, we have done extensive histological analyses of the liver as a function of time after MVE-2 or *C. parvum* administrations. These studies have revealed a large influx of mononuclear cells extravasating into the interstitium of the liver by 3 d (Wiltrott, R. H., and C. W. Reynolds, manuscript in preparation). This increase in mononuclear cell infiltration corresponded to the large increases in LGL number and NK activity (Tables I and II and Figs. 1-3). It therefore appears that the increase in LGL number and NK activity in the liver is a result of both proliferation and increased LGL localization. The major effect is most likely a result of an increased lymphocyte infiltration into the liver, possibly caused by the depot of MVE-2 in the liver (56).

There are several potentially important implications of BRM-induced LGL accumulation in nonlymphoid organs. First, these cells can be highly effective in inhibiting the formation of metastases in these organs.³ Second, an influx of LGL may also be of considerable relevance to the therapy of viral diseases. Stein-Streilein et al. (54) have demonstrated a compartmentalized augmentation of NK activity in the lungs as a result of influenza virus. This augmented NK activity may be important on the eventual resolution of virus infections. In fact, Bukowski et al. (57) have demonstrated that depletion of NK activity enhances virus-induced hepatitis and virus synthesis *in vivo*. Further, these authors have

noted enhanced tumor rejection and augmented NK activity in mice persistently infected with lymphocytic choriomeningitis virus (58). Cumulatively, these results suggest a potentially important role for tissue LGL in the resistance to metastases and viral diseases.

In summary, this study shows that the BRMs MVE-2 and *C. parvum* can induce greatly augmented NK activity in the liver. This increased liver-associated NK activity is due to an increase in LGL that have a surface phenotype similar, but not identical, to previously characterized murine LGL populations. The liver should therefore provide a source of LGL in high purity that can be used to study numerous aspects of activated LGL function and biology in the mouse. Finally, the observation of an increased tissue-associated NK activity that strongly correlates with our previous observations regarding a decrease in tumor metastases following BRM injection suggests that some BRMs can induce an increase in tissue-associated immune responses that may have direct therapeutic or clinical implications for neoplastic and/or virus-induced diseases.

Summary

Natural killer (NK) activity in the rat and human has been attributed to cells having the morphology of large granular lymphocytes (LGL). However, this association has been less clear in the mouse, largely because of difficulties in obtaining highly enriched populations of LGL from normal spleen and blood. We have previously observed that the administration of the biological response modifier (BRM) maleic anhydride divinyl ether (MVE-2) strongly augmented NK activity in lung and liver, and the augmented NK activity coincided with increased resistance to the formation of experimental metastases in these organs. The degree of NK augmentation was most striking in the liver, an unexpected and previously unreported observation. In the present study, both MVE-2 or *Corynebacterium parvum* induced a dramatic augmentation of liver NK activity, which reached maximum levels 3–5 d after treatment. This augmentation of NK activity in the liver coincided with a large increase in the number of lymphoid cells with the morphological characteristics of LGL that could be isolated from enzymatically digested suspensions of perfused liver. The yield of LGL per liver following BRM treatment corresponded to a 10–50-fold increase as compared to normal mice. LGL were purified from these enzymatically digested suspensions of perfused liver by depletion of adherent cells on nylon wool columns and subsequent enrichment for low-density lymphoid cells by fractionation on Percoll density gradients. The enrichment of LGL correlated with greatly increased NK activity against YAC-1. Conversely, the higher-density fractions were depleted of both LGL and NK activity. This increase in NK activity in the liver was suppressed by *in vivo* treatment with anti-asialo GM₁ (asGM₁) serum. This treatment also resulted in a corresponding reduction in both the total number and percentage of LGL. By flow cytometry analysis, the phenotype of the majority of these highly cytolytic LGL isolated from the livers of BRM-treated mice were asGM₁⁺, Thy-1⁺, Ly-5⁺, Qa-5⁺, Mac-1⁺, and Gma-1⁺, whereas these LGL were Ly-1⁻, Lyt-2⁻, L3T4⁻, and surface Ig⁻. We conclude that the livers of BRM-treated mice can provide a rich source of highly active mouse LGL that could be used for further characterization of this lymphocyte subset. Further,

these studies imply a potential for BRM therapy of neoplastic or viral diseases through augmentation of organ-associated immune responses.

We wish to acknowledge the excellent technical assistance of Ms. Patricia Urias, Ms. Robin Winkler, and Ms. Jill Coshun, as well as the excellent contributions of Mr. Llewellyn Mason and Mr. Kenneth Green, Jr. We appreciate the efforts of Mrs. Louise Shaw in typing the manuscript. We also thank Dr. Joost J. Oppenheim for his careful review of the manuscript. In addition, special thanks go to Mr. Roy Overton for his excellent assistance in performing the flow cytometry analyses.

Received for publication 23 July 1984.

References

1. Talmadge, J., K. Meyers, D. Prieur, and J. Starkey. 1980. Role of NK cells in tumor growth and metastasis in beige mice. *Nature (Lond.)* 284:622.
2. Hanna, N., and R. Burton. 1981. Definitive evidence that natural killer (NK) cells inhibit experimental tumor metastasis *in vivo*. *J. Immunol.* 121:1754.
3. Gorelik, E., R. H. Wiltrot, K. Okumura, S. Habu, and R. B. Herberman. 1982. Role of NK cells in the control of metastatic spread and growth of tumor cells in mice. *Int. J. Cancer.* 30:107.
4. 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-asGM₁ treated rats. *J. Immunol.* 131:1024.
5. Welsh, R. M. 1978. Cytotoxic cells induced during lymphocyte choriomeningitis virus infection in mice. I. Characterization of natural killer cell induction. *J. Exp. Med.* 148:163.
6. Welsh, R. M., Jr., R. M. Zinkernagel, and L. A. Hallenbeck. 1979. Cytotoxic cells induced during lymphocytic choriomeningitis virus infection of mice. *J. Immunol.* 122:475.
7. Fitzgerald, P. A., R. Evans, D. Kirkpatrick, and C. Lopez. 1983. Heterogeneity of human NK cells: comparison of effectors that lyse HSV-1 infected fibroblasts and K562 erythroleukemia targets. *J. Immunol.* 130:1663.
8. Timonen, T., and E. Saksela. 1980. Isolation of human natural killer cells by discontinuous gradient centrifugation. *J. Immunol. Methods.* 36:285.
9. Reynolds, C. W., T. 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.
10. Reynolds, C. W., S. O. Sharrow, J. R. Ortaldo, and R. B. Herberman. 1981. Natural killer activity in the rat. III. Analysis of surface antigens on LGL by flow cytometry. *J. Immunol.* 127:2204.
11. Reynolds, C. W., T. T. Timonen, H. T. Holden, C. T. Hanson, and R. B. Herberman. 1982. Natural killer (NK) cell activity in the rat. Analysis of effector cell morphology and effects of interferon on NK cell function in the athymic (nude) rat. *Eur. J. Immunol.* 12:577.
12. Reynolds, C. W., A. C. Denn III, T. Barlozzari, R. H. Wiltrot, 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.
13. Timonen, T., J. R. Ortaldo, and R. B. Herberman. 1982. Characteristics of human large granular lymphocytes and relationship to natural killer and K cells. *J. Exp. Med.* 153:569.

14. Ortaldo, J. R., S. O. Sharrow, T. T. Timonen, and R. B. Herberman. 1981. Analysis of surface antigens on highly purified human NK cells by flow cytometry with monoclonal antibodies. *J. Immunol.* 127:2401.
15. Farrum, E., and S. R. Targan. 1983. Identification of human natural killer soluble cytotoxic factors (NKCF) derived from NK-enriched lymphocytic populations: specificity of generation and killing. *J. Immunol.* 130:1252.
16. 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.
17. 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.
18. 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.
19. Tagliabue, A., A. D. Befus, D. A. Clark, and J. Bienenstock. 1982. Characteristics of natural killer cells in the murine intestinal epithelium and lamina propria. *J. Exp. Med.* 155:1785.
20. Leventon, G. S., S. S. Kulkarni, M. L. Meistrich, J. R. Newland, and A. R. Zanden. 1983. Isolation of murine small bowel intraepithelial lymphocytes. *J. Immunol. Methods.* 63:35.
21. Sjögren, H. O., and I. Hellstrom. 1965. Induction of polyoma specific transplantation antigenicity in Maloney leukemia cells. *Exp. Cell. Res.* 40:208.
22. Fischer, G. A. 1958. Studies of the culture of leukemia cells *in vitro*. *Ann. NY Acad. Sci.* 76:673.
23. Dunn, T. B., and M. J. Potter. 1957. A transplantable mast cell neoplasm in the mouse. *J. Natl. Cancer Inst.* 18:587.
24. Glynn, J. P., J. L. McCoy, and A. Fefer. 1968. Cross-resistance to the transplantation of syngeneic Friend, Moloney, and Rauscher virus-induced tumors. *Cancer Res.* 28:434.
25. Burton, R. C., S. P. Bartlett, V. Kumar, and H. J. Winn. 1981. Studies on natural killer (NK) cells. II. Serologic evidence for heterogeneity of murine NK cells. *J. Immunol.* 127:1864.
26. Stutman, O., C. J. Paige, and E. FeoFigarella. 1978. Natural cytotoxic (NC) cells against solid tumors in mice. I. Strain and age distribution and target susceptibility. *J. Immunol.* 121:1819.
27. Chirigos, M. A., and W. A. Stylos. 1980. Immunomodulatory effect of various molecular-weight maleic anhydride divinyl ethers and other agents *in vivo*. *Cancer Res.* 40:1967.
28. 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.
29. Richman, L. K., R. J. Klingenstein, J. A. Richman, W. Strober, and J. A. Berzofsky. 1979. The murine Kupffer cell. I. Characterization of the cell serving accessory function in antigen-specific T-cell proliferation. *J. Immunol.* 123:2602.
30. Mathieson, B. J., S. O. Sharrow, K. Bottomly, and B. J. Fowlkes. 1980. Ly 9, an alloantigenic marker for lymphocyte differentiation. *J. Immunol.* 125:2127.
31. Chirigos, M. A., E. Schlick, M. Piccoli, E. Read, K. Hartung, and A. Bartocci. 1982. Characterization of agents. *Adv. Immunopharmacol.* 2:669-677.
32. Kasai, M., T. Yoheda, S. Habu, Y. Maruyama, K. Ikumura, and R. Tokunaga. 1980. *In vivo* effect of anti-asialo GM₁ antibody on natural killer activity. *Nature (Lond.)* 291:334.

33. Young, W. W., Jr., S. Hakomori, J. M. Durdik, and C. S. Henney. 1980. Identification of ganglio-*N*-tetraosylceramide as a new marker for murine natural killer (NK) cells. *J. Immunol.* 124:199.
34. Ortaldo, J. R., and R. B. Herberman. 1984. Heterogeneity of natural killer cells. *Annu. Rev. Immunol.* 2:359.
35. Roder J. C., M. L., Lohmann-Matthes, W. Domzig, R. Kiessling, and O. Haller. 1979. A functional comparison of tumor killing by activated macrophages and natural killer cells. *Eur. J. Immunol.* 9:283.
36. Wiltrott, R. H., M. J. Brunda, and H. T. Holden. 1982. Variation in selectivity of tumor cell cytotoxicity by murine macrophages, macrophage-like cell lines, and NK cell. *Int. J. Cancer.* 30:335.
37. Kasai, M., J. C. Leclerc, F.-W., Shen, and H. Cantor. 1979. Identification of Ly 5 on the surface of "natural killer" cells in normal and athymic inbred mouse strains. *Immunogenetics.* 8:153.
38. Minato, N., L. Reid, and B. R. Bloom. 1981. On the heterogeneity of murine natural killer cells. *J. Exp. Med.* 154:750.
39. Chun, M., V. Pasanen, U. Hammerling, G. Hammerling, and M. K. Hoffman. 1979. Tumor necrosis factor induces a serologically distinct population of NK cells. *J. Exp. Med.* 150:426.
40. Lattime, E. C., G. A. Pecoraro, and O. Stutman. 1983. The activity of natural cytotoxic cells is augmented by interleukin 2 and interleukin 3. *J. Exp. Med.* 157:1070.
41. Koo, G., J. Jacobson, G. Hammerling, and U. Hammerling. 1980. Antigenic profile of murine natural killer cells. *J. Immunol.* 125:1003.
42. Pollack, S. B., M. R. Tam, R. C. Nowinski, and S. L. Emmons. 1979. Presence of T cell-associated surface antigens on murine NK cells. *J. Immunol.* 123:1818.
43. Mattes, J., M. Jules, 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.
44. Mathieson, B. J., R.-W. Shen, A. Brooks, and W. M. Leiserson. 1982. MG-1, identification of an early differentiation marker for bone marrow cells in a monocytic/granulocytic lineage. *Adv. Exp. Med. Biol.* 155:201.
45. Holmberg, L. A., T. A. Springer, and K. A. Ault. 1981. Natural killer activity in the peritoneal exudates of mice infected with *Listeria monocytogenes*: characterization of the natural killer cells by using a monoclonal rat anti-murine macrophage antibody (M1/70). *J. Immunol.* 127:1792.
46. Sun, D., and M. L. Lohmann-Matthes. 1982. Functionally different subpopulations of mouse macrophages recognized by monoclonal antibodies. *Eur. J. Immunol.* 12:134.
47. Pierres, A., P. Naquet, A. Van Agthoven, F. Bekkhoucha, F. Denizot, Z. Mishal, A. Schmitt-Verhulst, and M. Pierres. 1984. A rat anti-mouse T4 monoclonal antibody (H129.19) inhibits the proliferation of Ia-reactive T cell clones and delineates two phenotypically distinct (T4⁺, Lyt-2,3⁻, and T4⁻, Lyt-2,3⁺) subsets among anti-Ia cytolytic T cell clones. *J. Immunol.* 132:2775.
48. Shortman, K., A. Wilson, R. Scollay, and W.-F. Chen. 1983. Development of large granular lymphocytes with anomalous, nonspecific cytotoxicity in clones derived from Ly-2⁺ T-cells. *Proc. Natl. Acad. Sci. USA.* 80:2728.
49. Brooks, C. G. 1983. Reversible induction of natural killer cell activity in cloned murine cytotoxic T-lymphocytes. *Nature (Lond.)* 305:155.
50. Lattime, E. C., G. A. Pecoraro, and O. Stutman. 1981. Natural cytotoxic cells against

- solid tumors in mice. III. A comparison of effector cell antigenic phenotype and target cell recognition structures with those of NK cells. *J. Immunol.* 126:2011.
51. Warner, N. L., A. S. Tai, and D. L. Lewis. 1982. Heterogeneity of NK cells of mouse and man. *In* Natural Killer Cells. Vol. 4: Human Cancer Immunology. B. Serrou, C. Rosenfeld, and R. B. Herberman, editors. Elsevier/North-Holland Biomedical Press. Amsterdam. 249–264.
 52. Herberman, R. B., M. E. Nunn, and H. T. Holden. 1978. Low density of Thy 1 antigen on mouse effector cells mediating natural cytotoxicity against tumor cells. *J. Immunol.* 121:304.
 53. Tam, M. R., S. L. Emmons, and S. B. Pollack. 1980. Analysis and enrichment of murine natural killer cells with fluorescent-activated cell sorter. *J. Immunol.* 124:650.
 54. 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.
 55. Biron, C. A., and R. M. Welsh. 1982. Proliferation and role of natural killer cells during viral infection. *In* NK Cells and Other Natural Effector Cells. R. B. Herberman, editor. Academic Press, Inc., New York. 493.
 56. Carrano, R. A., R. K. Kinoshita, A. R. Imondi, and J. D. Iuliucci. 1981. MVE-2: preclinical pharmacology and toxicology. *In* Augmenting Agents in Cancer Therapy. E. M. Hersh, M. A. Chirigos, and M. J. Mastrenghelo, editors. Raven Press, New York. 345–372.
 57. Bukowski, J. F., B. A. Woda, S. Habu, K. Okumura, and R. M. Welsh. 1983. Natural killer cell depletion enhances virus synthesis and virus-induced hepatitis *in vivo*. *J. Immunol.* 131:1531.
 58. Bukowski, J. F., C. A. Biron, and R. M. Welsh. 1983. Elevated natural killer cell-mediated cytotoxicity, plasma interferon and tumor cell rejection in mice persistently infected with lymphocytic choriomeningitis virus. *J. Immunol.* 154:840.