HETEROGENEITY OF NATURAL KILLER CELLS IN THE MOUSE*

By JOHN A. LUST, VINAY KUMAR,‡ ROBERT C. BURTON,§ SCOTT P. BARTLETT, AND MICHAEL BENNETT

From the Department of Pathology, Boston University School of Medicine, Boston, Massachusetts 02118; and the Transplantation Unit, Massachusetts General Hospital, Boston, Massachusetts 02114

It is now well established that many mammalian species possess leukocytes that are able to lyse a variety of tumors in vitro without prior immunization (1, 2). These cells have been termed natural killer (NK)[1] cells. Characterization of the NK cells reactive against different lymphoid and solid tumor targets has revealed that they share certain properties but differ in others. For example, NK activity of spleen cells against the lymphoma YAC-1 is very low in mice injected with the bone-seeking isotope ^{89}Sr and in mice <3 wk of age (3). In contrast, NK activity against EL-4 lymphoma cells is relatively unaffected in mice treated with ^{89}Sr and is present in mice 5 d old (4). However, both the NK (YAC-1) and NK (EL-4) activities are stimulated by interferon inducers administered in vivo (4). Stutman and coworkers (5, 6) have characterized an NK cell active against solid tumors that they have termed a natural cytotoxic (NC) cell. One notable difference between NC cells and the typical NK cell active against lymphoid tumors is that NC cells remain functional after incubation at 37°C for as long as 6 d, whereas NK cells are labile at 37°C for as few as 6 h under the same culture conditions (5, 6). These results suggest that NK cells in general may be a heterogenous population. For purposes of discussion, we will designate all non-T, non-B, and non-macrophage cells active in the natural cytotoxicity assay as NK cells and will propose a tentative classification of NK cells.

To evaluate the concept of heterogeneity of NK cells, we have used a variety of methods to distinguish between NK cells reactive against a panel of tumors, both lymphoid and nonlymphoid. Our studies, presented here, indicate that NK cells in fresh spleen cell suspensions can be classified into two broad categories on the basis of their susceptibility to suppression by treatment with ^{89}Sr, presence of NK alloantigens, and activity in beige (*bg/bg*) mice.

Materials and Methods

Mice. (C57BL/6 × DBA/2)F_1 (B6D2F1), B10.D2, CBA/J, C57BL/6J, C57BL/6J-*bg/bg*, and BALB/c mice were purchased from either The Jackson Laboratory (Bar Harbor, Maine) or Charles River Breeding Laboratories (Wilmington, Mass.). Neonatal mice were obtained

* Supported by grants CA-21401, CA-15369, CA-17800, CA-20044, AM-07055, and HL-18646 from the National Institutes of Health, Bethesda, Md.

‡ Cancer Research Scholar of the American Cancer Society, Massachusetts Division.

§ John Mitchell Crouch Fellow of the Royal Australasian College of Surgeons.

[1] *Abbreviations used in this paper:* C, complement; C:H, cold:hot; E:T, effector:target; meth A, methylcholanthrene; N, normal; NC, natural cytotoxic; NK, natural killer; T, thymus.

J. Exp. Med. © The Rockefeller University Press · 0022-1007/81/08/0306/12 $1.00
Volume 154 August 1981 306–317

through breeding in our animal facility. The mice were 6–12 wk of age unless otherwise indicated, and mice of either sex were used.

Tumor Cells. The tumor cell lines YAC-1, Cl.18, and WEHI-164.1 have been described in detail elsewhere (7, 8) and were maintained in vitro by culturing in RPMI-1640 (Grand Island Biological Co., Grand Island, N. Y.), with 10% fetal calf serum, penicillin and streptomycin, L-glutamine, sodium pyruvate, and nonessential amino acids (complete RPMI-1640 medium). FLD-3, a Friend virus-induced erythroleukemia of BALB/c origin (H-2^d), was obtained from Dr. Richard A. Steeves (Albert Einstein School of Medicine, New York) and maintained in complete RPMI-1640. MPC-11 cells, a mineral oil-induced myeloma of BALB/c origin (H-2^d) were obtained from the Salk Cell Distribution Center, San Diego, Calif.

NK Cell Assay. The assays used have been described in detail previously (4, 8–11). Approximately 5×10^6 tumor cells were labeled with 100 μCi $Na_2\ ^{51}CrO_4$ (New England Nuclear, Boston, Mass.) in 0.5 ml of complete RPMI-1640 for 1–1.5 h, or by overnight incubation at 5 μCi/ml. The cells were washed three times and diluted to a final concentration of 1×10^5–2×10^5 tumor cells per ml. 1×10^4–2×10^4 targets in 0.1 ml of complete RPMI-1640 medium were placed in wells of Microtest II plates (Falcon Labware, Div. of Becton, Dickinson & Co., Oxnard, Calif.) with varying numbers of fresh spleen cells in 0.1 ml of complete RPMI-1640 medium. Generally, each spleen cell suspension was plated at four different effector to target cell (E:T) ratios ranging from 100:1 to 1.5:1. Cells at each ratio were plated in triplicate or quadruplicate. The incubation periods were 4–16 h with YAC-1, 12–16 h with WEHI-164.1, 8–18 h with Cl.18, and 24 h with FLD-3. These time periods were selected because preliminary experiments indicated that significant lysis of these targets required assays of these durations. After incubation, the plates were centrifuged at 200 *g* and 0.1 ml of supernate was harvested from each well and counted in a Packard Prias (Packard Instrument Co., Inc., Downers Grove, Ill.) or a Beckman 4000 Auto-γ counter (Beckman Instruments, Inc., Fullerton, Calif.). The mean percent specific cytotoxicity was calculated as follows:

$$\text{mean percent cytotoxicity} = {}^{51}\text{Cr, cpm}\ \frac{\text{experimental} - \text{spontaneous (medium)}}{\text{maximal } (H_2O) - \text{spontaneous}} \times 100.$$

The variation between the replicates was <5%. Spontaneous release values depended on the tumor target and the assay time but did not exceed one-third of the maximum release value in the experiments reported herein. Where single figures are given, they are taken from the corresponding linear part of the E:T vs. percent cytotoxicity curve.

Cold competition experiments were performed as described in detail previously (4, 11) by using E:T ratios of 50:1 for YAC-1 and 25:1 for WEHI-164.1 and adding varying numbers of cold (i.e., unlabeled) targets, generally at cold to hot (^{51}Cr-labeled) cell ratios (C:H) of 50:1, 25:1, and 12.5:1. Note that C:H ratios of 50:1 were not exceeded because nonspecific inhibition, particularly with tumor cells, becomes significant at high C:H ratios (11). ^{51}Cr release values with labeled and cold targets were never significantly different from spontaneous release values with labeled targets alone.

Mouse Pretreatments. Heat-killed *Corynebacterium parvum* organisms (Wellcome Laboratories, Raleigh, N. C.) were injected intraperitoneally (2.1 mg/mouse). Cyclophosphamide was obtained from Mead Johnson and Co., Evansville, Ind.; mice were injected intraperitoneally with 300 mg/kg body weight. 6–8-wk-old mice were injected with 100 μCi ^{89}Sr (Oak Ridge National Laboratory, Oak Ridge, Tenn.) intraperitoneally and used 4–6 wk after the injection. Mice were irradiated in a small-animal irradiator (Gammacell 40, Atomic Energy of Canada Ltd., Commercial Products, Ottawa, Ontario, Canada; dual ^{137}Cs sources, 136 rad/min). The details of these procedures have been described earlier (4).

Cell Pretreatments. Treatment of spleen cells with CE anti-CBA, anti-Nk-1.2, serum (9, 10) and pretested rabbit complement (C) was performed by the following protocol. Erythrocytes were removed from a fresh spleen cell suspension by hypotonic shock and 20×10^6 lymphocytes from this suspension were treated with a 1:40 dilution of anti-Nk-1.2 for 30 min at 37°C in complete RPMI-1640 without fetal calf serum. The cells were washed once in complete RPMI-1640 and treated with C (1:8 dilution) for 30 min at 37°C in serum-free medium. The cells were washed once and then adjusted to the appropriate concentration for an NK cell assay. The viability of spleen cells after treatment with anti-Nk-1.2 plus C was 95–97%.

Results

NK Activity of Spleen Cells from ^{89}Sr-treated Mice. NK activity against YAC-1, Cl.18, and MPC-11 was markedly reduced in B6D2F1 and B10.D2 mice pretreated with the bone-seeking isotope ^{89}Sr (Table I). In contrast, NK reactivity against FLD-3 and WEHI-164.1 was not different from spleen cells obtained from ^{89}Sr-treated B10.D2 mice as compared with untreated controls. When spleen cells from ^{89}Sr-treated B6D2F1 mice were used as effectors, NK activity against WEHI-164.1 was modestly reduced and, in some cases, NK (FLD-3) activity was greatly reduced.

To ascertain whether the reduced NK (FLD-3) activity seen in some B6D2F1 mice was due to lack of functional NK effector cells, as is the case with NK (YAC-1) (7), or due to the presence of suppressor cells, a cell mixture experiment was performed (Fig. 1). The NK (FLD-3) activity of normal spleen cells could be greatly reduced by the addition of spleen cells from ^{89}Sr-treated mice. This finding is in accord with our previous observations relating to NK (EL-4) cells. NK (EL-4), like NK (FLD-3), is usually normal in ^{89}Sr-treated mice, but when reduced, the lowering can be accounted for by the presence of suppressor cells (4, 12). On the other hand, NK (YAC-1) is always lowered in ^{89}Sr-treated mice, and similar cell mixture experiments have consistently failed to demonstrate suppressor cells in our hands (4, 12).

Effect of Anti-Nk-1.2 plus C on NK Activity. The NK activity in fresh spleen cells from BALB/c mice against YAC-1 and Cl.18 was markedly reduced by prior treatment of the spleen cells with anti-Nk-1.2 plus C (Table II). In contrast, NK activity against FLD-3 and WEHI-164.1 was largely resistant to treatment with anti-

TABLE I

Effect of ^{89}Sr Treatment on NK Activity

Strain	Target*	Pretreatment	Percent cytotoxicity at E:T of				
			100:1	50:1	25:1	6.25:1	1.56:1
B10.D2	YAC-1	None	21	13	8	—	—
		^{89}Sr‡	9	4	5	—	—
	Cl.18	None	15	—	11	8	—
		^{89}Sr	6	—	9	4	—
	FLD-3	None	—	—	25	18	10
		^{89}Sr	—	—	34	28	20
	WEHI-164.1	None	—	—	65	55	29
		^{89}Sr	—	—	68	66	49
B6D2F1	YAC-1	None	68	59	45	—	—
		^{89}Sr	2	−3	−1	—	—
	Cl.18	None	23	—	16	3	—
		^{89}Sr	2	—	7	1	—
	MPC-11	None	—	25	18	11	—
		^{89}Sr	—	4	3	3	—
	FLD-3	None	—	—	22	20	14
		^{89}Sr	—	—	5	12	8
	FLD-3	None	—	—	20	15	4
		^{89}Sr	—	—	15	13	8
	WEHI-164.1	None	—	—	67	60	48
		^{89}Sr	—	—	56	45	35

* The incubation period was 12 h with YAC-1 and WEHI-164.1, 18 h with Cl.18, and 24 h with FLD-3.

‡ Mice pretreated with ^{89}Sr were given one 100 μCi injection intraperitoneally. The mice were tested 4–8 wk after the injection for NK activity.

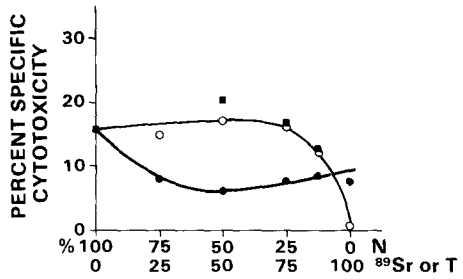

FIG. 1. The NK activity of mixtures (totaling 1×10^6 lymphocytes) of normal (N) spleen cells with normal thymus (T; ○) or with spleen cells from ^{89}Sr-treated (^{89}Sr) mice (●) was measured. 1×10^6 lymphocytes and 2×10^4 ^{51}Cr-labeled FLD-3 cells were incubated in wells of microtiter plates for 24 h. Closed squares (■) indicate the NK activity of unmixed normal spleen cells at E:T of 50:1 (100% N), 25:1 (50% N), 12.5:1 (25% N), and 6.25:1 (12.5% N).

TABLE II

Effect of Anti-Nk-1.2 plus C on NK Activity

Target	Strain	Pretreatment*	Percent cytotoxicity at E:T of		
			50:1	25:1	12.5:1
YAC-1	BALB/c	C	48	33	19
		Anti-Nk-1.2 + C	7	4	−1
	B6D2F1	C	40	23	11
		Anti-Nk-1.2 + C	4	1	2
	CBA	C	38	20	11
		Anti-Nk-1.2 + C	0	0	0
FLD-3	BALB/c	C	25	20	9
		Anti-Nk-1.2 + C	21	17	9
	B6D2F1	C	18	20	12
		Anti-Nk-1.2 + C	23	16	9
WEHI-164.1	BALB/c	C	51	47	34
		Anti-Nk-1.2 + C	39	32	23
	CBA	C	72	71	41
		Anti-Nk-1.2 + C	79	68	58
Cl.18	BALB/c	C	25	14	—
		Anti-Nk-1.2 + C	4	3	—
MPC-11	BALB/c	C	15	—	—
		Anti-Nk-1.2 + C	0	—	—

* 20×10^6 lymphocytes were treated with a 1:40 dilution of anti-Nk-1.2 for 30 min at 37°C, washed, and treated with a 1:8 dilution of rabbit C for 30 min at 37°C. The cells were washed and then adjusted to the appropriate concentration for an NK cell assay.

Nk antibody plus C. It should be noted however, that in some experiments with anti-Nk-1.2 plus C, a twofold reduction in NK activity against WEHI-164.1 has been observed. However, this must be compared with the total abolition of NK activity against YAC-1, which is generally effected by this treatment (Table II).

NK Activity in Beige Mice. C57BL/6 mice homozygous for the beige mutation (*bg/bg*) have reduced NK activity against YAC-1 lymphoma cells in short-term ^{51}Cr release assays (13). In contrast, NK activity against FLD-3 and WEHI-164.1 is normal in such mice (Table III). The low NK (YAC-1) seen in beige mice can be partially and in some experiments totally corrected by a prolongation of the assay period to 16 h (Table III), which suggests that any defect in NK activity against FLD-3 and

TABLE III
NK Activity in Beige Mice

Experiment	Strain	Assay time	Pretreatment	Percent cytotoxicity at E:T of 100:1		
				YAC-1*	WEHI-164.1	FLD-3
		h				
1	C57BL/6 +/+	*	None	27	76	57
	C57BL/6 *bg/bg*	*	None	3	78	61
2	C57BL/6 +/+	16	C	24	100	—
			Anti-Nk-1.2 + C	1	88	—
	C57BL/6 *bg/bg*	16	C	15	95	—
			Anti-Nk-1.2 + C	0	84	—

* The incubation period was 12 h with YAC-1 and 18 h with WEHI-164.1 and FLD-3 in experiment 1.

TABLE IV
NK Activity of Spleen Cells from Mice Pretreated with C. parvum or Cyclophosphamide

Strain	Pretreatment*	Percent cytotoxicity‡		
		YAC-1	FLD-3	WEHI-164.1
C57BL/6	None	35	—	26
	C. parvum	59	—	60
BALB/c	None	25	26	42
	C. parvum	53	37	61
	Cyclophosphamide	10	ND§	47
B6D2F1	None	34	14	50
	Cyclophosphamide	10	5	66

* Mice received intraperitoneally 2.1 mg *C. parvum* or 300 mg/kg body weight of cyclophosphamide.

‡ The mean percent cytotoxicity of cells from C57BL/6 and BALB/c mice was measured at an E:T ratio of 50:1. The mean percent cytotoxicity of cells from B6D2F1 mice was measured at an E:T ratio of 25:1. The incubation period was 12 h with YAC-1 and WEHI-164.1, and 24 h with FLD-3.

§ Not done.

WEHI-164.1 can be masked by the longer duration of the assay. If this were so, no distinction could be made between NK (YAC-1) and NK (FLD-3) or NK (WEHI-164.1) on the basis of data from beige mice. That this is not the case was clarified when we tested the ability of anti-Nk-1.2 plus C-treated spleen cells of beige mice to lyse YAC-1 cells in a long-term assay. Table III shows that although some Nk (YAC-1) reactivity can be seen in beige spleen cells, when the effectors and targets are incubated for 16 h, all such activity is mediated by Nk-1.2 antigen-positive cells, whereas the NK reactivity against WEHI-164.1 in beige mice is, as expected, mediated by cells not sensitive to anti-Nk-1.2 plus C treatment.

NK Activity of Spleen Cells from Mice Pretreated with C. parvum or Cyclophosphamide. The NK activity against FLD-3 and WEHI-164.1 was enhanced by pretreatment with *C. parvum* similar to the increase seen with YAC-1 (Table IV). Pretreatment with cyclophosphamide, an agent known to suppress NK activity, reduced NK activity in B6D2F1 mice against FLD-3 and YAC-1. The cytotoxic activity against WEHI-164.1,

however, was generally found to be resistant to the suppressive effect of cyclophosphamide. Pretreatment with cyclophosphamide reduces the number of nucleated cells obtained from a spleen by 60–75% (unpublished observations). Therefore, cyclophosphamide reduced the total number of NK cells in the spleen against all targets, but it further decreased the frequency of NK cells active against FLD-3 and YAC-1. In contrast, the frequency of NK cells reactive against WEHI-164.1 was not affected.

Effect of Lethal Irradiation on NK Activity in B6D2F1 Mice. We were interested in testing the effect of short-term lethal irradiation on NK activity against FLD-3 and WEHI-164.1 because the YAC-1 NK cell has already been shown to be radio resistant (3). B6D2F1 mice were lethally irradiated (800 rad) and 3 d later, their pooled spleen cells had retained NK activity against YAC-1 and WEHI-164.1 but had lost the ability to lyse FLD-3 (Table V). As with cyclophosphamide, short-term lethal irradiation reduced the total number of nucleated cells in the spleen by 80–90%. Therefore, the frequency of NK cells active against YAC-1 and WEHI-164.1 was normal but that against FLD-3 was decreased.

Effect of Age on NK Activity. As with YAC-1 (1, 2), the NK activity in B6 mice <3 wk old against FLD-3 was reduced when compared with B6 adult mice (Table VI). In contrast, spleen cells from (B6 × C3H)F_1 mice 1 d old were able to lyse WEHI-164.1 as well as adult mice. There was no lytic activity against YAC-1 cells in 1-d-old (B6 × C3H)F_1 mice.

Competitive Inhibition of NK Activity against YAC-1 and WEHI-164.1. To test whether the antigens recognized by NK cells against different targets were similar, we carried out cold competition studies (Table VII). These results indicate that the NK cell that kills YAC-1 recognizes target structures that are expressed on YAC-1 and Cl.18, whereas the NK cell that lyses WEHI-164.1 is largely specific for that target. There was some inhibition of YAC-1 lysis by FLD-3 and WEHI-164.1, suggesting either

TABLE V

NK Activity of Spleen Cells from Lethally Irradiated B6D2F1 Mice

Pretreatment*	Percent Cytotoxicity‡		
	YAC-1	FLD-3	WEHI-164.1
None	50	27	50
800 rad, −3 d	64	1	57

* 10 B6D2F1 mice were lethally irradiated (800 rad) and their pooled spleen cells were used 3 d later in an NK cell assay.

‡ Mean percent cytotoxicity at an E:T ratio of 25:1 with YAC-1, WEHI-164.1, and FLD-3.

TABLE VI

Effect of Age on NK Activity

Age	Assay time	Strain	Percent cytotoxicity at E:T of 100:1		
			YAC-1	WEHI-164.1	FLD-3
	h				
1 d	18	(B6 × C3H)F_1	0	65	0
8 wk	18	(B6 × C3H)F_1	24	57	ND
2 wk	24	C57BL/6	—	—	−1
8 wk	24	C57BL/6	—	—	17

TABLE VII

Competitive Inhibition of NK Activity against YAC-1 and WEHI-164.1

Experiment	Cold cell type	Percent cytotoxicity against*					
		YAC-1 (C:H)			WEHI-164.1 (C:H)		
		50:1	25:1	12.5:1	50:1	25:1	12.5:1
1	YAC-1	−1	6	12	29	30	30
	FLD-3	32	47	46	58	54	46
	WEHI-164.1	24	32	37	10	24	34
2	YAC-1	1	5	10	—	—	—
	Cl.18	13	25	28	—	—	—

* E:T ratio was 50:1 for all C:H ratios with labeled YAC-1, and 25:1 for all C:H ratios with labeled WEHI-164.1; mean percent cytotoxicity without cold targets was 50 with YAC-1 and 45 with WEHI-164.1 in experiment 1, and 35 with YAC-1 in experiment 2.

that a minor subpopulation of NK cells that lyse YAC-1 recognizes target determinant(s) shared between the three tumors, or that the target structures on FLD-3 and WEHI-164.1 resemble to some extent (cross-react with) those on YAC-1. FLD-3 did not inhibit lysis of the WEHI-164.1 target by NK cells, and YAC-1 competed poorly, in a plateau fashion, as compared with cold WEHI-164.1. This minor degree of inhibition of NK-mediated WEHI-164.1 lysis by YAC-1 can be interpreted in the same two ways as the inhibition of lysis of YAC-1 by WEHI-164.1 (see above). However, the fact that some reduction of NK activity against WEHI-164.1 can often be achieved by treatment of spleen cells with anti-Nk-1.2 plus C (Table II and our unpublished observations) suggests that WEHI-164.1 might be susceptible to lysis by two different NK cell types: the major activity is WEHI-164.1 specific, preserved in neonatal, beige, and ^{89}Sr-treated mice, and is resistant to anti-Nk-1.2 plus C treatment, and a minor component is mediated by the NK cell that lyses YAC-1, which recognizes a separate shared target structure on the two tumors.

Discussion

NK cells remain a somewhat poorly defined class of cells that may have important functions in host defense and immunoregulation (1, 2, 14, 15). At present, the best definition is that NK cells, which are not classical T cells, B cells, or macrophages, are capable of lysing a variety of tumor and some normal cells in vitro (1). Whether all the cells that are capable of NK function in vitro belong to a single class in terms of lineage, origin, and differentiation remains an open question. Some indications that natural killing may be mediated by a heterogenous population of cells has been provided by the studies of Stutman et al. (5), Paige et al. (6), and Lohman-Matthes and Domzig (16, 17). Although cold target competition studies and adsorption of NK cells over tumor cell monolayers suggest a certain selectivity in target cell recognition (18), these by themselves only indicate a possible heterogeneity of recognition structures, rather than heterogeneity in the class of cells bearing the recognition units. Here we provide a clear indication that NK cells in fresh spleen cell suspensions can be classified into two distinct categories based on three major criteria: susceptibility to the reduction of activity in mice treated with ^{89}Sr, reduced activity in mice with

the beige mutation, and the susceptibility to lysis by an anti-Nk-1.2 alloantiserum and complement.

Our earlier studies had indicated that NK activity against YAC-1 but not EL-4 lymphoma was reduced in ^{89}Sr-treated mice (4, 12). An analysis of low NK (YAC-1) activity in ^{89}Sr-treated mice has indicated that an intact bone marrow microenvironment may be essential for the full functional differentiation of NK (YAC-1) cells (7). By this criterion, NK cells could be classified into those that are marrow dependent, such as NK cells reactive against YAC-1, RL ♂-1, MPC-11, and Cl.18, whereas those reactive against FLD-3,[2] EL-4, and WEHI-164.1 can differentiate from splenic stem cells in the absence of an intact bone marrow. The NC cell activity against BALB/c methylcholanthrene (meth A)-induced sarcoma is also minimally affected in ^{89}Sr-treated mice (19). It is interesting to note that this notion of marrow dependence is also supported by studies in estrogen-treated mice. Mice treated with high doses of estrogens develop osteopetrosis, and these mice are strikingly similar to ^{89}Sr-treated mice with respect to NK activity (20, 21). NK (YAC-1) activity is reduced, it is not boosted significantly by an interferon inducer (pI:pC), and yet NK (or NC) activity against the BALB/c meth A sarcoma is unaffected (19). Preliminary results obtained in osteopetrotic humans (lacking normal bone marrow) also show a lowered NK reactivity against HSV-1-infected fibroblasts (C. Lopez, personal communication), supporting the concept of marrow dependence of certain human NK cells. It should be pointed out that although NK reactivity against EL-4 and FLD-3 is often normal in most ^{89}Sr-treated mice, it is low in some instances. However, the reduction in activity against these targets in ^{89}Sr-treated mice is generally triggered by the "activation" of suppressor cells by interferon inducers (12) and not due to a lack of functional NK cells.

The experiments with anti-Nk-1.2 alloantiserum provide independent evidence for the subclassification of NK cell activity into two classes. The antiserum used has been defined in detail elsewhere (9, 10). Under the conditions used here, this serum is NK cell specific and stains only 2–5% of spleen cells (22; unpublished observations in a fluorescence-activated cell sorter analysis). It seems more than coincidental that marrow-dependent NK cells, i.e., those that lyse YAC-1, Cl.18, and MPC-11, are also Nk-1.2 antigen positive, as defined by significant reduction in NK activity by pretreatment with anti-Nk-1.2 plus C. The marrow-independent NK cells, including those that lyse meth A sarcoma (O. Stutman, personal communication), are resistant to such a treatment and may, by this criteria, be considered Nk-1.2 antigen negative. We cannot state with certainty that NK (FLD-3) and NK (WEHI-164.1) are totally devoid of Nk-1.2 antigens. The possibilities of very low antigen density and resistance to complement-mediated lysis must also be considered. It may be recalled that Ly-5 antigen is expressed on several cell types, including T cells, NK cells, and B cells. However, B cells cannot be lysed by anti-Ly-5 serum plus C under the usual conditions (23). Although resistance to C-mediated lysis cannot be ruled out completely, it seems to be an unlikely explanation because NK (WEHI-164.1) can be lysed by heterologous anti-mouse and anti H-2 antisera and C.[3] Nevertheless, positive selection studies will be required to answer this question definitively.

[2] Lust, J. A., V. Kumar, and M. Bennett. Lysis by natural killer cells and rejection by irradiated mice of Friend virus induced erythroleukemia cells. Manuscript in preparation.

[3] Burton, R. C., S. P. Bartlett, V. Kumar, and H. J. Winn. Studies on natural killer (NK) cells. III. Serological evidence for heterogeneity of NK cells. Manuscript in preparation.

Studies in beige mice further support and extend the notion of NK cell heterogeneity developed from the aforementioned experiments. The function of the Nk antigen-positive, marrow-dependent class of NK cells is impaired in beige mice (Table III). However, the lytic ability of beige spleen cells can be considerably restored by prolongation of the NK assay (Table III). Thus, the beige mutation appears to cause more of a delay in, rather than lack of, killing. Cells that mediate such delayed killing of targets of the YAC-1 type are still distinguishable from those that are responsible for the lysis of WEHI-164.1 cells, which normally take longer to be lysed, by being susceptible to anti-Nk-1.2 plus C. It is interesting to note that beige mice and ^{89}Sr-treated mice seem to have defects affecting the same class of cells, and there are some similarities between beige mice and ^{89}Sr-treated mice. These include a relative resistance to boosting by interferon and a normal frequency of target-binding cells (7, 13). Nevertheless, the basis of the NK defect in ^{89}Sr-treated and beige mice must be quite different, because beige mice seem to have a stem cell defect (13). Thus their "NK defect" can be adoptively transferred to normal mice by bone marrow cells, and they themselves can be largely "cured" by normal hemopoietic stem cell transplants. On the other hand, the low NK (YAC-1) of ^{89}Sr-treated mice cannot be cured by a normal bone marrow cell infusion, and spleens of ^{89}Sr-treated mice do possess normal stem cells for NK (YAC-1) (7, 24).

One difference between the two categories of NK cells is the length of time required for target cell lysis. In general targets such as YAC-1, RL ♂-1, and MPC-11, can be lysed in a shorter period of time (4–6 h), whereas tumors such as WEHI-164.1, FLD-3, and meth A take a longer period of incubation for optimal lysis (12–48 h). Could the differences be related merely to the length of the assay? We do not believe this to be the case because spleen cells from ^{89}Sr-treated mice and anti-Nk-1.2 plus C-treated spleen cells remain deficient in their ability to kill YAC-1 cells, even if the assay is prolonged to 16–24 h, the usual time necessary to observe the lysis of FLD-3 and WEHI-164.1. With beige mice, the delayed killing of YAC-1 is mediated by Nk-positive cells as discussed above. Nevertheless, it could be argued that tumors such as FLD-3 or WEHI-164.1 may be efficient inducers of interferons in vitro. The interferon could then recruit new NK cells from their precursors, even if the mature NK cells had been depleted by anti-Nk plus C or by ^{89}Sr treatment. That this is not the case is indicated by our preliminary studies (25), in which equivalent amounts of interferon were generated in 24-h culture supernates of spleen cells with a variety of tumor cells, including YAC-1, FLD-3, and some NK-insensitive tumor cells such as L1210, but no correlation was found between the induction of interferon in vitro and the lysis of the tumor cell line.

On the basis of the data discussed above, we propose that NK cells be classified into two categories: NK-A cells would include the Nk antigen-positive, marrow-dependent NK cells, and NK-B would designate Nk antigen-negative cells, which have normal activity in ^{89}Sr-treated and beige mice (Table VIII). The NC cells described by Stutman et al. (5) would thus fall into the NK-B category. It appears that NK-A cells that react largely against lymphoid targets are a much more homogenous group as compared with NK-B cells (Table VIII). NK-B cells all share certain common characteristics, but also differ from each other in several respects. For example, although NK cells reactive against the meth A sarcoma (19), WEHI-164.1, and FLD-3 are all marrow independent and Nk antigen negative, NK (FLD-3) differ

TABLE VIII
Classification of NK Cells

Type	Characteristics	Target selectivity
NK-A	Lytic activity reduced in ^{89}Sr-treated and beige mice; susceptible to lysis by anti-Nk-1.2 + C	YAC-1, Cl.18, RL ♂-1, MPC-11*
NK-B (includes NC cells)	Normal in ^{89}Sr-treated mice, normal in beige mice; resistant to lysis by anti-Nk-1.2 + C	FLD-3, WEHI-164.1, meth A*

* Classification of RL ♂-1 and Meth A was based on data in references 4 and 19, respectively.

from the rest. Unlike the other cells in the NK-B category, NK (FLD-3) are absent or nonfunctional in newborn mice and are markedly reduced in frequency in mice given lethal whole body irradiation (−3 d) or cyclophosphamide. NK (WEHI-164.1) and NC cells reactive against meth A sarcoma seem not to differ much among themselves. Further investigations may reveal subsets among the two major categories we have defined. At present, however, similarities between the cells classified as NK-B seem significant enough to group them together. This distinction between NK-A and NK-B can be applied solely to the NK activity of normal fresh spleen cells, because boosting with interferon or in vitro culture is known to change the physical characteristics, antigen expression, and target selectivity of NK-like cells (26, 27).

It is tempting to speculate that the NK-B cells that are Nk antigen negative and marrow independent may be precursors of NK-A cells; the transition from NK-B to NK-A may require an intact marrow. In previously reported experiments (4), we have found that NK (EL-4) appears earlier than NK (YAC-1) in the spleens of lethally irradiated mice transplanted with normal syngeneic precursor bone marrow cells. However, such a schema is by no means proven, and delineation of the relationships between NK-A and NK-B cells must await further investigations. In closing, it may be pointed out that the existence of two or more classes of NK cells does not necessarily argue for or against heterogeneity of putative NK cell receptors or their clonal distribution. T and B cells, which are two distinct classes of immunocompetent cells, are known to share similar idiotypic determinants (28). It is therefore conceivable that distinct classes of cells that can mediate natural cytotoxicity may use similar recognition structures if common cell surface antigens are present on their target cells.

Summary

Mice were treated with the bone-seeking isotope, ^{89}Sr, cyclophosphamide, and short-term lethal irradiation in vivo, and murine spleen cells were treated with anti-Nk-1.2 plus complement (C) in vitro. Fresh spleen cell suspensions from the above groups and from beige and neonatal mice were subsequently tested for natural killer (NK) cell activity against a panel of lymphoid and nonlymphoid tumor cell targets. NK cell reactivities against YAC-1, MPC-11, and Cl.18 tumors were markedly and consistently reduced in (*a*) mice treated with ^{89}Sr, (*b*) spleen cells treated with anti-Nk-1.2 plus C, and (*c*) C57BL/6 *bg/bg* mice. In contrast, NK activities against FLD-3 and WEHI-164.1 tumors were usually normal in mice treated with ^{89}Sr, in beige mutant mice, and in spleen cells after treatment with anti-Nk-1.2 antibody and C. It appears, therefore, that two major groups of NK cells exist in fresh mouse spleen cell suspensions. NK-A cells are marrow dependent, Nk antigen positive, and deficient

in beige mice; these lyse YAC-1, MPC-11, and Cl.18 tumors. NK-B cells, which are responsible for the lysis of WEHI-164.1 and FLD-3, are Nk antigen negative, marrow independent, and unaffected by the *bg/bg* mutation. Other features of NK-B cells suggest that these NK cells, although they share the characteristics mentioned above, differ among themselves especially with respect to age of maturation and susceptibility to cyclophosphamide and total body irradiation. The NK-B group may therefore include subsets that remain to be defined.

We thank Kathleen Donovan for excellent secretarial assistance in the preparation of this manuscript and Dr. C. Lopez and Dr. O. Stutman for communicating their unpublished results.

Received for publication 30 March 1981.

References

1. Kiessling, R., and H. W. Wigzell. 1979. An analysis of the murine NK cell as to structure, function and biological relevance. *Immunol. Rev.* **44:**165.
2. R. B. Herberman, editor. 1980. Natural Cell-Mediated Immunity against Tumors. Academic Press, Inc., New York. 1321 pp.
3. Kiessling, R., P. S. Hochman, O. Haller, G. M. Shearer, H. Wigzell, and G. Cudkowicz. 1977. Evidence for a similar or common mechanism for natural killer cell activity and resistance to hemopoietic grafts. *Eur. J. Immunol.* **7:**655.
4. Kumar, V., E. Luevano, and M. Bennett. 1979. Hybrid resistance to EL-4 lymphoma cells. I. Characterization of natural killer cells which lyse EL-4 cells and their distinction from marrow-dependent natural killer cells. *J. Exp. Med.* **150:**531.
5. Stutman, O., C. Paige, and E. F. Figarella. 1979. Natural cytotoxic cells against solid tumors in mice. I. Strain and age distribution and target cell susceptibility. *J. Immunol.* **121:** 1819.
6. Paige, C., E. F. Figarella, M. J. Cuttito, A. Cahan, and O. Stutman. 1978. Natural cytotoxic cells against solid tumors in mice. II. Some characteristics of the effector cells. *J. Immunol.* **121:**1827.
7. Kumar, V., J. Ben-ezra, M. Bennett, and G. Sonnenfeld. 1979. Natural killer cells in mice treated with ^{89}strontium: normal target-binding cell numbers but inability to kill even after interferon administration. *J. Immunol.* **123:**1832.
8. Burton, R. C., D. Grail, and N. L. Warner. 1978. Natural cytotoxicity of hemopoietic cell populations against murine lymphoid tumors. *Br. J. Cancer.* **37:**806.
9. Burton, R. C., and H. J. Winn. Studies on natural killer (NK) cells. I. NK cell specific antibodies in CE anti-CBA serum. *J. Immunol.* **126:**1985.
10. Burton, R. C. 1980. Alloantisera selectively reactive with NK cells: characterization and use in defining NK cell classes. *In* Natural Cell-Mediated Immunity against Tumors. R. B. Herberman, editor. Academic Press, Inc., New York. 19.
11. Chism, S. E., R. C. Burton, D. L. Grail, P. M. Bell, and N. L. Warner. 1977. In vitro induction of tumor-specific immunity. VI. Analysis of immune response by cellular competitive inhibition: limitations and advantages of the technique. *J. Immunol. Methods.* **16:**245.
12. Luevano, E., V. Kumar, and M. Bennett. Hybrid resistance to EL-4 lymphoma cells. II. Association between loss of hybrid resistance and detection of suppressor cells after treatment of mice with ^{89}Sr. *Scand. J. Immunol.* In press.
13. Roder, J. C. 1979. The beige mutation in the mouse. I. A stem cell predetermined impairment in natural killer cell function. *J. Immunol.* **123:**2168.

14. Haller, O., A. Orn, M. Gidlund, and H. Wigzell. 1980. In vivo activity of murine NK cells. *In* Natural Cell-mediated Immunity against Tumors. R. B. Herberman, editor. Academic Press, Inc., New York. 1105.
15. Hochman, P. S., and G. Cudkowicz. 1979. Suppression of natural cytotoxicity by spleen cells of hydrocortisone-treated mice. *J. Immunol.* **123:**968.
16. Lohmann-Matthes, M.-L., and W. Domzig. 1979. Antibody dependent cellular cytotoxicity (ADCC) against tumor cells. I. Cultivated bone marrow macrophages kill tumor targets. *Eur. J. Immunol.* **9:**261.
17. Domzig, W., and M.-L. Lohmann-Matthes. 1979. Antibody dependent cellular cytotoxicity (ADCC) against tumor cells. II. The promonocyte identified as an effector cell. *Eur. J. Immunol.* **9:**267.
18. Herberman, R. B., M. F. Nunn, and D. H. Lavrin. 1975. Natural cytotoxic reactivity of mouse lymphoid cells against syngeneic and allogeneic tumors. II. Characterization of effector cells. *Int. J. Cancer.* **16:**230.
19. Stutman, O., E. F. Figarella, C. J. Paige, E. C. Lattime. 1980. Natural cytotoxic (NC) cells against solid tumors in mice: general characteristics and comparison to natural killer (NK) cells. *In* Natural Cell-mediated Immunity against Tumors. 1980. R. B. Herberman, editor. Academic Press, Inc., New York. 187.
20. Seaman, W. E., T. D. Gindhart, J. S. Greenspan, M. A. Blackman, and N. Talal. 1979. Natural killer cells, bone, and bone marrow: studies in estrogen-treated mice and in congenitally osteopetrotic (*mi/mi*) mice. *J. Immunol.* **122:**2541.
21. Seaman, W. E., T. C. Merigan, and N. Talal. 1979. Natural killing in estrogen-treated mice responds poorly to poly I·C despite normal stimulation of circulating interferon. *J. Immunol.* **123:**2903.
22. Burton, R. C., S. P. Bartlett, V. Kumar, and H. J. Winn. 1981. Heterogeneity of murine natural killer (NK) cells. *Fed. Proc.* **40:**1006.
23. Scheid, M. P., and D. Triglia. 1979. Further description of the Ly-5 system. *Immunogenetics.* **9:**423.
24. Levy, E., M. Bennett, V. Kumar, P. Fitzgerald, and S. Cooperband. 1980. Adoptive transfer of spleen cells from mice treated with radioactive strontium: suppressor cells, natural killer cells, and "hybrid resistance" in recipient mice. *J. Immunol.* **124:**611.
25. Kumar, V., J. A. Lust, A. Gifaldi, M. Bennett, and G. Sonnenfeld. 1981. Natural killer (NK) cell lysis of Friend erythroleukemia cells (FLD-3) does not require interferon (IFN) induction. *Fed. Proc.* **40:**1092.
26. Kiessling, R., E. Erikson, L. Hallenbeck, and R. Welsh. 1980. A comparative analysis of the cell surface properties of activated vs. endogenous mouse natural killer cells. *J. Immunol.* **125:**1551.
27. Bartlett, S. P., and R. C. Burton. 1981. Cultured natural killer (NK) cells. *Fed. Proc.* **40:** 1092.
28. Binz, H., and H. Wigzell. 1975. Shared idiotypic determinants on B-lymphocytes and T-lymphocytes reactive against same antigenic determinants. I. Demonstration of similar or identical idiotypes on IgG molecules and T-cell receptors with specificity for same alloantigens. *J. Exp. Med.* **142:**197.