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Generation of natural killer cells from bone marrow precursors *in vitro*

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SUMMARY

A simple and highly reproducible bone marrow culture system for the generation of cytolytically active NK cells from immature precursors in the bone marrow is described. The NK cells can be generated with various sources of IL-2, including Con A-conditioned medium, supernatants from IL-2-producing cell lines and recombinant IL-2. Neither IL-1, IL-3 nor α/β interferon induced significant cytotoxicity in bone marrow cells. Identification of the cytotoxic cells as NK cells was based on their phenotypic characteristics (aGM1$^+$, Thy 1$^\pm$, Ly 1$^-$2$^-$. Ia$^-$, RIL-2$^\pm$, H2$^+$), as well as their spectrum of target specificity. The deliberate addition of peripheral blood mononuclear cells as a source of mature NK cells and elimination of cells expressing markers specific for mature NK cells indicated that the generated NK cells were descendants of precursors of NK cells harboured in the bone marrow and not derived from mature NK cells contaminating the bone marrow preparations. Thus, it was shown that not only functionally active NK cells but also their precursors are highly dependent on IL-2 for differentiation and growth. This culture system should be helpful in studying the origin of NK cells in relation to other cell lineages as well as the regulation of the maturation of NK cells from their precursors.

INTRODUCTION

Natural killer (NK) cells have as general characteristics no requirement for priming, rapid onset of killing and a broad spectrum of reactivity (Herberman, 1982). These properties make the NK cells a possible part of basic defence mechanisms, and experimental evidence supports a role for NK cells in the resistance to tumour cells and various infectious micro-organisms (Talmadge *et al.*, 1980; Kalland & Forsberg, 1981; Bukowski *et al.*, 1983; Hatcher & Kuhn, 1982), as well as in the regulation of haematopoietic homeostasis (Hansson, Kiessling & Anderson, 1981; Hansson *et al.*, 1982).

While much progress has been made in studies on the regulation of the cytotoxic activity of NK cells, information concerning their lineage of origin as well as their regulation at the stem cell level is severly lacking. A potential useful approach for examining these fundamental questions would be to study the development of NK cells from their precursors *in vitro*. NK cells originate from bone marrow and are highly dependent on a continuous bone marrow function (Haller *et al.*, 1979; Seaman *et al.*, 1978; Kumar *et al.*, 1979). The present paper describes a simple and highly reproducible bone marrow culture system that enables the generation of functionally active mature NK cells from cytolytically inactive precursors in mouse bone marrow.

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MATERIALS AND METHODS

Animals

Four to 8-week-old C57BL/6 (B6) or BALB/c mice were obtained from Gamle Bomholtgaard, Ry, Denmark, and were kept at our institute by strict brother-to-sister mating. Sprague–Dawley rats were obtained from Gamle Bomholtgaard and were used at 3–5 months of age.

Cell preparations

The mice were killed by cervical dislocation and bone marrow cells were obtained by flushing tibia and femur with ice-cold RPMI-1640 with 10% fetal calf serum (FCS, Gibco Biocult, Paisley, Renfrewshire, and Flow Laboratories, Irvine, Ayshire) through a 27-gauge needle. All cells were kept on ice until they were cultured. Spleen cells were prepared by teasing the spleen through a stainless steel mesh and red blood cells removed by hypotonic shock treatment (Kalland & Forsberg, 1983). Peripheral blood was obtained from the retro-orbital venous plexus and mononuclear cells isolated on Lymphoprep (Nyegaard & Co., Oslo, Norway) (Kalland, Alm & Stålhandske, 1985). Rats were killed with ether and each thymus was dissected free from adhering tissue. A single cell suspension of thymus cells was prepared by gently teasing the thymus with fine forceps in the same medium as mentioned above.

Cell culture

All cultures were made up in RPMI-1640 medium supplemented

with 25 mM Hepes buffer, 2 mM glutamin, penicillin/streptomycin (100 U/μg/ml). FCS at various concentrations as well as supernatants from Con A-stimulated rat spleen cell cultures prepared as described elsewhere (Kalland, 1985) were added as indicated in the Results. Supernatants from the following cell lines were used as indicated: WEHI-3, a murine T-cell line known to produce constitutive high levels of interleukin-3 (IL-3), was obtained from Dr R. Palacios, Basel Institute of Immunology, Switzerland (Lee, Hapel & Ihle, 1982). The EL4·IL2 subclone of the EL4 murine thymoma producing high levels of interleukin-2 (IL-2) when stimulated with phorbol myristate acetate (PMA) was obtained from American Type Culture Collection (ATCC, Rockville, MD) and stimulated as described elsewhere (Farrar *et al.*, 1980). The Gibbon T-cell line MLA 144 constitutively producing IL-2 was kindly provided by Dr C. Borrebaeck, University of Lund (Rabin *et al.*, 1981). Supernatants from the cell lines were used without further purification. Recombinant IL-2 (rIL-2) was a generous gift from Cetus Immune Corporation (Emeryville, CA), and its characteristics have been described elsewhere (Rosenberg *et al.*, 1984). Recombinant interleukin-1 (rIL-1, specific activity 6×10^6 U/mg) was generously provided by Dr Lomedico, Hoffmann La Roche Inc., Nutley, NJ (Lomedico *et al.*, 1984) and was used at a concentration three times that giving maximal proliferation in the thymocyte stimulation assay. A partially purified preparation of mouse α and β interferon was purchased from Calbiochem-Behring Corp., La Jolla,CA (sp. act. 10^5 U/mg) and used at concentrations giving optimal stimulation of spleen NK activity. The cells were cultured for 1–14 days in an atmosphere of 7% CO_2 at 37° in 2 ml culture medium in Nunc 24-well multidish culture plates (cat. no. 168357, Nunc A/S, Roskilde, Denmark). One ml of the medium was replaced with fresh medium every third day. The cells were harvested by vigorous pipetting, and in some instances the remaining adherent cells were recovered by 5 min incubation in ice-cold PBS with 0·02% EDTA (w/v, Gibco).

Culture of target cells

The murine cell lines YAC-1, P815 and B16 and the human leukaemia K-562 were cultured as described elsewhere (Kalland *et al.*, 1985). Allogenic blasts cells were obtained by activation of BALB/c spleen lymphocytes with 5 μg/ml concanavalin A (Con A, Sigma Chemicals, Poole, Dorset) for 3 days and were further propagated in culture medium containing IL-2 as described in a previous paper (Kalland, 1985).

NK cell assay

Effector cell generated in bone marrow cultures or fresh spleen cells were tested for NK activity against target cells in a standard 4-hr ^{51}chromium-release assay as described in detail previously (Kalland & Forsberg, 1981). All assays were run at effector to target cell ratios of 100:1, 50:1 and 25:1. As similar results were obtained with different effector to target ratios, only the former are shown. The data are presented as the percentage specific cytotoxicity, which was determined as:

$$\frac{\text{test c.p.m.} - \text{spontaneous c.p.m.}}{\text{total c.p.m.} - \text{spontaneous c.p.m.}} \times 100.$$

Culture-generated cytotoxic cells were also tested in a single cell assay slightly modified from that originally described by Grimm & Bonavida (1979). In brief, 10^5 nylon wool non-adherent cultured cells and 2×10^5 YAC-1 target cells were mixed in 200 μl in conical microflex tubes (Kontes Co., Vineland, NJ). The cells were incubated for 5 min at 30°, were spun at 500 *g* for 5 min, and were further incubated at 30° for 10 min. The supernatant was carefully poured off, and 50 μl RPMI-1640 with 10% FCS were added. The cells were resuspended by gentle pipetting twice with a 50-μl automat-pipet, and 50 μl molten agarose (Sigma Chemicals, type I: VII blended 1:5) at 39° were added directly to the resuspended pellet. The cells were then smeared onto agarose precoated glass slides, and were allowed to gel at room temperature for 1–2 min before being incubated for 3 hr at 37° submerged in RPMI-1640 medium with 10% FCS. The slides were then stained with trypan blue (Sigma Chemicals), washed three times in phosphate-buffered saline (PBS), and were fixed in 0·5% formaldehyde in PBS. The percentage of lymphocytes binding a target cell was calculated by counting 200 lymphocytes, and the frequency of bound lymphocytes that had lysed their target by counting at least 50 conjugates.

Cell surface markers

The presence of cell surface markers on precursors of the cytotoxic cells generated in the bone marrow cultures was examined by treating fresh bone marrow cells with appropriate predetermined dilutions of the antibodies described below and complement (C, low-toxic rabbit complement, Cederlane Laboratories Ltd, Ontario, Canada; final dilution 1:8). The cells were adjusted to 10^7/ml and incubated with antibodies for 45 min on ice, washed once and further incubated for 45 min at 37° with rabbit C. The cells were then washed twice in RPMI-1640 medium with 10% FCS and cultured as described. Cytotoxicity against YAC-1 cells was determined after culture for 3 days.

The phenotype of the effector cells was examined in a similar manner by treatment of bone marrow cells cultured for 3 days with antibodies and C. Cell numbers of effector cells were not readjusted after antibody and C treatment to prevent artifactural enrichment of surviving cells. The following monoclonal antibodies were used: HO-134 [mouse anti-Thy 1.2, from ATCC, developed by Marshak-Rothstein *et al.* (1979)], 53-7.313 [rat anti-Ly 1, from ATCC, established by Ledbetter & Herzenberg (1979)], 19/178c [mouse anti-Ly 2.2, from ATCC, described by Hämmerling, Hämmerling & Flaherty (1979)], 7D4 (rat anti-mouse IL-2-receptor (RIL-2), generously provided by Dr T. Malek, NIH, Bethesda, MD (Malek, Robb & Shevach, 1983), M1/42.3.9.8 [rat anti-H2, from ATCC, developed by Bhattacharya, Dorf & Springer (1981)] and M5/114.15.2 [rat anti-$IA^{b,d,k}$, $IE^{b,d}$, from ATCC, described by Kennett (1980)]. In addition, a rabbit antiserum to the glycosphinolipid GM1 (aGM1) (Kasai *et al.*, 1981) was purchased from Wako Pure Chemical Industries Ltd, Osaka, Japan, and used at a final dilution of 1:50.

RESULTS

NK activity is low but detectable in the bone marrow of adult mice (Fig. 1). However, when bone marrow cells are cultured with Con A-conditioned medium from spleen cell cultures, strong cytotoxic activity against YAC-1 cells is generated (Fig. 1). After an initial lag-phase of 2 days, cytotoxic activity rapidly emerges at Day 3 of culture, and can be detected throughout the 2-week culture period studied. Parallel studies at the single cell

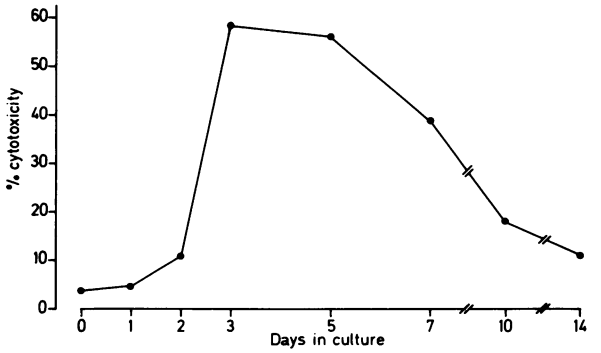

Figure 1. Cytotoxic activity against YAC-1 cells of C57BL/6 bone marrow cells cultured for various times with 20% conditioned medium from Con A-stimulated rat spleen cells. Effector:target of 100:1.

level revealed an increase in the number of cultured cells capable of binding and lysing YAC-1 cells (Fig. 2).

Basic features of the culture system with regard to cell density and feeder cell requirements were systematically investigated (Table 1). While significant cytotoxic activity could reliably be generated with 6×10^5 bone marrow cells per culture well, maximal activity was obtained with 3×10^6 cells per well. At the lower cell densities, the addition of rat thymocytes, which alone generated no cytotoxic activity, improved the results. However, high concentrations of bone marrow cells circumvented the need for feeder cells.

During the initial work with the culture system, it was found that different batches of FCS varied considerably in their ability to support the generation of cytotoxic cells from bone marrow (Table 2). The critical importance of the serum source was absolute and was not only related to differences in the kinetics of the induction of cytotoxic activity (data not shown). All experiments in the present report were performed with Batch B of fetal calf serum (Table 2).

In order to identify the active substance(s) in Con A-conditioned medium used to generate the cytotoxic cells, supernatants from various cell lines, known to produce high levels of certain lymphokines, as well as some DNA recombinant preparations, were tested (Fig. 3). Supernatants known to

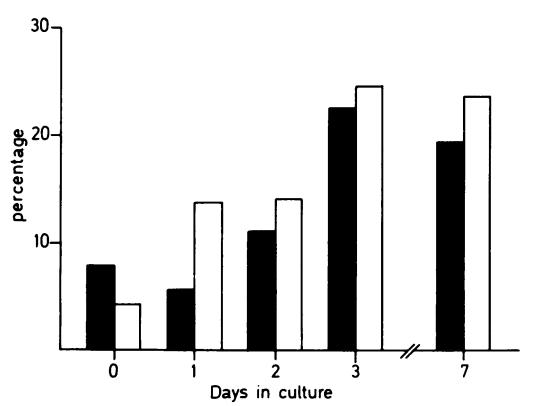

Figure 2. Percentage of nylon wool non-adherent cells generated in bone marrow culture for various times with 10% supernatant from PMA-stimulated EL4.IL2 cells able to bind YAC-1 target cells (■); (□) indicates the percentage of target binding cells able to lyse their targets as determined in a single cell assay.

Table 1. Effect of cell density and feeder cells on generation of cytotoxicity

Cells per well ($\times 10^{-6}$)		
Bone marrow	Thymocytes	% cytotoxicity
6	—	52·3
3	—	69·1
1·5	—	47·0
0·6	—	10·4
0·3	—	3·5
0·1	—	0·9
3	8	41·6
3	4	60·4
3	2	70·2
3	1	65·3
0·6	8	22·4
0·6	4	29·0
0·6	2	15·3
0·6	1	12·5
—	8	0·9
—	4	1·6
—	2	1·5
—	1	0·9

The cells were cultured with 10% supernatant from PMA-stimulated EL4·IL2 cells for 3 days and tested for cytotoxicity against YAC-1 cells at an effector:target ratio of 100:1.

Table 2. The ability of different batches of fetal calf serum to support the generation of cytotoxic cells in bone marrow cultures

	% cytotoxicity*			
Batch	2%†	5%	10%	20%
A	5·6	10·7	19·4	18·5
B	20·7	54·0	81·2	65·3
C	6·5	15·9	31·5	29·3
D	8·4	16·3	27·4	20·7
E	0·0	3·8	7·8	8·4

* Effector:target ratio of 100:1.
† Bone marrow cells were cultured with 10% supernatant from PMA-stimulated EL4.IL2 cells in the presence of 2-20% FCS from different batches.

contain high levels of IL-2 (conditioned medium, EL4-IL2 and MLA 144 cell lines) gave excellent results in the assay. Neither IL-3-containing supernatants from the WEH-3 cell line nor IL-1 were able to support the induction of cytotoxic cells. A partially purified preparation of α and β mouse interferon gave a weak and inconsistent cytotoxic activity after 3 days of culture. Finally, it was unequivocally shown that IL-2 was neccessary, as well as sufficient, to generate cytotoxic cells from inactive precursors in the bone marrow by using rIL-2 which induced strong cytotoxic activity (Fig. 3). Cultures without IL-2 generated no cytotoxicity.

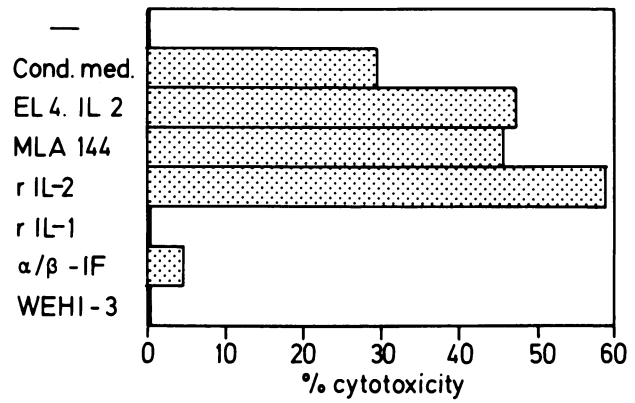

Figure 3. Cytotoxic activity against YAC-1 cells of bone marrow cells cultured for 3 days without additions (—), with 20% Con A-conditioned medium (cond. med.), with 10% supernatant from PMA-stimulated EL4.IL2 cells, with 10% supernatant from MLA 144 cells, with 50 U/ml rIL-2, with 90 U/ml rIL-1, with 500 U/ml α/β interferon, or with 10% supernatant from the WEHI-3 cell line. Effector:target ratio of 100:1.

In order to characterize the IL-2-dependent bone marrow-derived cytotoxic effector cell, two approaches were taken. Firstly, the cytotoxic activity of the bone marrow-derived cells was compared with that of fresh spleen NK cells on several target cells with different susceptibilities to different cytotoxic cells. The results are summarized in Fig. 4, showing that the culture-generated cells exerted strong cytotoxic activity against the NK-susceptible targets YAC-1 and B16, but only weak or insignificant cytotoxic activity against the NK-resistant P815 mastocytoma, the human NK target K-562 or Con A-activated allogenic blast cells. Moreover, the cytotoxic profile against these targets of the cultured bone marrow cells was closely similar to that of fresh spleen NK cells. Secondly, the phenotype of the cytotoxic cells from the bone marrow cultures was determined by elimination of cells bearing various cell surface structures with specific antibodies and C. These experiments indicated that the phenotype of the culture-generated cytotoxic cell was aGM1$^+$, Thy 1.2$^\pm$, Ly 1$^-$2$^-$, Ia$^-$, RIL-2$^\pm$, H2$^+$ (Table 3). This phenotype is similar to that of NK cells from spleen, with the exception that a larger proportion of the cultured bone marrow cytotoxic cells expressed Thy 1 and receptor for IL-2.

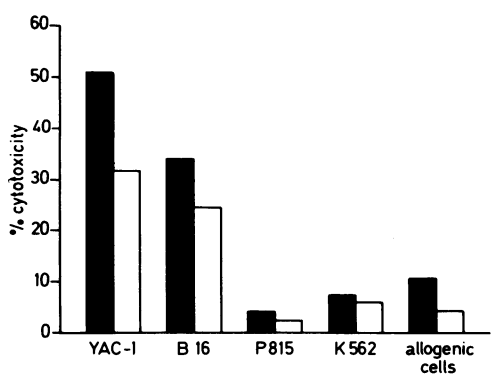

Figure 4. Cytotoxic activity against various target cells of bone marrow cells cultured for 3 days with 10% supernatant from the PMA-stimulated EL4.IL2 cell line (■) or fresh spleen NK cells (□). Effector:target ratio of 100:1.

Table 3. Cell surface markers on precursor cells and effector cells of bone marrow-generated cytotoxic cells and spleen NK cells

		% cytotoxicity*		
Antibody†	Specificity	Precursor cells	Effector cells	Spleen cells
—		61	52	34
nms		55	49	31
aGM1		52	3	1
HO-134	Thy 1.2	60	20	21
53–7.313	Ly 1	58	48	29
19/178c	Ly 2.2	56	40	30
7D4	IL2 receptor	38	25	25
M1/42.3.9.8	H2	3	2	1
M5/114.15.2	IA/IE	51	50	30

* Effector:target ratio of 100:1.

† Descriptions of the antibodies are given in the Materials and Methods. The cells were treated with antibodies and C, either before culture (precure cells) or after 3 days of culture in the presence of 10% PMA-stimulated EL4.IL2 supernatant (effector cells), and tested for cytotoxic activity against YAC-1 cells.

However, taken together, these data are quite conclusive in identifying the cytotoxic cells generated in the culture system as closely related to, if not identical with, NK cells.

Attempts to identify the precursor of the culture-generated NK cell in a similar manner by selective depletion of cells bearing certain cell surface markers with antibody and C before culture of the cells were only partially successful. Of the markers tested, only RIL-2 and H2 were apparently expressed on the NK cell precursor.

In order to estimate the effect of a possible contamination of mature NK cells from peripheral blood in the bone marrow preparation, 10% peripheral blood mononuclear cells were deliberately added to the bone marrow cells. The effect on the

Table 4. Effect of contamination with peripheral blood mononuclear cells and treatment with aGM1 antibodies and C on cytotoxic activity of cultured bone marrow cells

Treatment	Added cells	Day	% cytotoxicity
nms	–	1	8
nms	–	3	51
nms	+	1	22
nms	+	3	65
aGM1	–	1	6
aGM1	–	3	48
aGM1	+	1	6
aGM1	+	3	46

Peripheral blood mononuclear cells were added to the bone marrow cells where indicated and were treated with nms or aGM1 antibodies and C before culture. The cells were harvested on Day 1 or Day 3 and tested for cytotoxic activity against YAC-1 cells at an effector:target ratio of 100:1.

generation of cytotoxicity with or without pretreatment with antibodies to aGM1 and C is shown in Table 4. When peripheral blood mononuclear cells were added, an increase in cytotoxicity was already observed by Day 1, which was completely eliminated by pretreatment with aGM1 and C. These data indicate that, under the conditions used here, contamination with mature NK cells is apparantly of no significant importance, and that the generated NK cells indeed arise from precursors in the bone marrow.

DISCUSSION

Several lines of evidence clearly indicate that NK cells originate from precursors in the bone marrow. (i) Transfer of bone marrow cells from mice with high NK activity can confer high NK activity to recipient mice with low NK activity (Haller *et al.*, 1979). (ii) NK-like clones can be established from long-term bone marrow cultures (Young, Okumura & Moore, 1985). (iii) Destruction of bone marrow by ^{89}strontium or oestradiol-17β lowers the NK activity of mice (Seaman *et al.*, 1978; Kumar *et al.*, 1979). (iv) Permanent inhibition of NK activity by exposure of neonatal mice to diethylstilbestrol is due to effects of the bone marrow (Kalland, 1984). Thus, bone marrow cells are the ultimate source of NK precursor/stem cells. With the recent documentation that mature NK cells express receptors for and can be driven to proliferation by IL-2 (Suzuki *et al.*, 1983; Trinchieri *et al.*, 1984), we used a bone marrow culture system to explore the possibility of inducing maturation of NK cell precursors with IL-2. We were able to show that culture of bone marrow cells with conditioned medium from Con A-stimulated spleen cells or supernatants from various cell lines producing IL-2 generated cytotoxic activity. After an initial lag-phase, strong cytotoxic activity was detected at Day 3 of culture. While neither IL-1, IL-3 nor α/β-interferon was able to induce any cytotoxic activity, the essential role of IL-2 was confirmed using rIL-2 which alone induced excellent cytotoxicity. The target cell specificity as well as phenotype of the effector cell strongly argue that the culture-derived cells are NK cells. While NK-sensitive targets were easily killed, the NK-resistant cell line P815 was resistant. Since IL-2 is the active substance in the culture system, it would not be surprising if cytotoxic cells with similarities to the recently described lymphokine-activated killer cells were generated (Grimm *et al.*, 1982, 1983). However, the P815 cells are sensitive to these killer cells, and the failure of T-cell specific antibodies to affect NK maturation argues against a possible IL-2 driven differentiation of lymphokine-activated killer cells as well as differentiation of mature T cells to NK-like cells (Brooks, 1983). Moreover, it is unlikely that the bone marrow-derived NK cell is related to the natural cytotoxic (NC) cell described by Stutman, Paige & Figarella (1979) since these cells generally require a longer incubation time to excert their cytotoxic function, and moreover are aGM1-negative. The NC cell can also be kept in long-term culture in the presence of IL-3, which was without effect in the culture system described in the present report (Djeu *et al.*, 1983).

NK cells are heterogenous with respect to surface markers (Minato, Reid & Bloom, 1981). While all mature NK cells have been found to express aGM1, a variable proportion express Thy 1 (Kasai *et al*, 1981; Minato *et al.*, 1981). Elimination of Thy 1-positive cells reduced the cytotoxic activity by about 50%, which is somewhat higher than that seen with spleen NK cells. Also, a higher proportion of functionally active cells expressed detectable levels of RIL-2. These discrepancies to spleen NK cells can probably best be explained by the selection of cells due to culture conditions. Thus, while target cell specificity as well as the uniform existence of aGM1 indicate that we are dealing with NK cells, it cannot be excluded that some subpopulations of NK cells are not represented. It should also be noted that we made no attempts to quantify the expression of the individual surface markers present on the culture-generated NK cell, since all antibodies were used in saturating concentrations.

It has been suggested that NK cell maturation proceeds through two distinct steps, one of which requires an intact bone marrow microstructure (Kumar *et al.*, 1979). Destruction of bone marrow with ^{89}strontium is accompanied by the inability of adoptively transferred bone marrow cells to mature to lytically active NK cells. An intermediate NK cell, however, is present but is incapable of lysing target cells even after stimulation with interferon. Since we have been able to generate fully competent NK cells *in vitro*, the effect of ^{89}strontium can be suggested to involve either the production of IL-2 or an accessory cell that is functionally active in our culture system.

A question of fundamental importance is whether the cytotoxic cells generated from bone marrow cultures in the presence of IL-2 are true descendants of immature precursors of NK cells are merely due to multiplication of pre-existing NK cells. The lag of the cytotoxic activity in bone marrow, paralleled by a burst of generation of new target binding cells at Day 3 of culture, favours the former explanation. Pretreatment of bone marrow cells before culture with aGM1 and C had no effect on the generation of cytotoxicity. Moreover, the deliberate addition of a cell population containing mature NK cells induced an early peak of NK activity at Day 1, which was abrogated by elimination of the aGM1-expressing cells. Thus, it is judged as highly unlikely that the culture system simply expands, pre-existing NK cells harboured in the bone marrow.

Generation of NK-like cells from bone marrow was reported by Lohman-Matthes (Lohman-Matthes, Domzig & Roder, 1979) whose cultures required a monocytic growth factor and were difficult to reproduce. These cells had the characteristics of promonocytes. Recently, two groups have reported success in generating cytotoxic cells from bone marrow cultures with Con A-conditioned medium with similarities to the cytotoxic cell described by us (Koo, Peppard & Mark, 1984; Klimpel *et al.*, 1985). Several discrepancies between these systems and the present, both with regard to kinetics of induction and characteristics of the effector cell, are probably due to the various sources of IL-2 used, since Con A-conditioned medium also contains several other factors that may influence the final outcome of the culture. The data presented based on rIL-2 show that not only mature NK cells but also their precursors are highly dependent on IL-2 for maturation and growth. The culture conditions described in the present report are well-defined, highly reproducible, and should enable the investigation of fundamental unresolved questions regarding the biology of the NK cell, such as its lineage or origin, as well as the regulation of its differentiation from immature precursors.

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REFERENCES

BHATTACHARYA A., DORF M.E. & SPRINGER T.A. (1981) A shared alloantigenic determinant on Ia antigens encoded by the I-A and I-E subregions: Evidence for I region gene duplication. *J. Immunol.* **127,** 2488.

BROOKS C.G. (1983) Reversible induction of natural killer cell activity in cloned murine cytotoxic T lymphocytes. *Nature (Lond.)*, **305,** 155.

BUKOWSKI J.F., WODA B.A., HABU K., OKUMURA K. & WELSH R.M. (1983) Natural killer cell depletion enhances virus synthesis and virus-induced hepatitis *in vivo*. *J. Immunol.* **131,** 1531.

DJEU J.Y., LANZA E., PASTORE S. & HAPEL A.J. (1983) Selective growth of natural cytotoxic but not natural killer effector cells in Interleukin-3. *Nature (Lond.)*, **306,** 788.

FARRAR J.J., FULLER-FARRAR J., SIMON P.L., HILFIKER M.L., STADLER B.M. & FARRAR W.L. (1980) Thymoma production of T cell growth factor (Interleukin-2). *J. Immunol.* **125,** 2555.

GRIMM E.A. & BONAVIDA B. (1979) Mechanism of cell mediated cytotoxicity at the single cell level. Evidence for first-order kinetics of T cell mediated cytolysis and for heterogeneity of the lytic rate. *J. Immunol.* **123,** 2870.

GRIMM E.A., MAZUMDER A., ZHANG H.Z. & ROSENBERG S.A. (1982) Lymphokine activated killer cell phenomenon. I. Lysis of natural killer resistant fresh solid tumur cells by interleukin-2 activated autologous human peripheral blood lymphocytes. *J. exp. Med.* **155,** 1823.

GRIMM E.A., RAMSEY K.M., MAZUMDER A., WILSON D.J., DJEU D.Y. & ROSENBERG S.A. (1983) Lymphokine activated killer cell phenomenon. II. Precursor phenotype is serologically distinct from peripheral T lymphocytes, memory cytotoxic thymus-derived lymphocytes and natural killer cells. *J. exp. Med.* **157,** 884.

HALLER O., KIESSLING R., ÖRN A. & WIGZELL H. (1979) Generation of natural killer cells: an autonomous function of the bone marrow. *J. exp. Med.* **145,** 1411.

HÄMMERLING G., HÄMMERLING U. & FLAHERTY L. (1979) Qat-4 and Qat-5, new murine T-cell antigens governed by the Tla region and identified by monoclonal antibodies. *J. exp. Med.* **150,** 108.

HANSSON M., BERAN M., ANDERSSON B. & KIESSLING R. (1982) Inhibition of *in vitro* granulopoiesis by autologous allogenic human NK cells. *J. Immunol.* **129,** 126.

HANSSON M., KIESSLING R. & ANDERSON B. (1981) Human fetal thymus and bone marrow contain target cells for natural killer cell. *Eur. J. Immunol.* **11,** 8.

HATCHER F.M. & KUHN R.E. (1982) Destruction of *Trypanosoma cruzi* by natural killer cells. *Science*, **218,** 295.

HERBERMAN R.B. (ed.) (1982) *NK Cells and Other Natural Effector Cells.* Academic Press, New York.

KALLAND T. (1980) Reduced natural killer cell activity in mice after neonatal exposure to diethylstilbestrol. *J. Immunol.* **124,** 1297.

KALLAND T. (1984) Exposure of neonatal female mice to diethylstilbestrol persistently impairs NK activity through reduction of effector cells at the bone marrow level. *Immunopharmacol.* **7,** 127.

KALLAND T. (1985) Inhibition of T lymphocyte activation by Estramustine: dose-dependent interference with IL-2 production and later proliferation events. *Immunopharmacol.* **9,** 65.

KALLAND T., ALM G. & STÅHANDSKE T. (1985) Augmentation of mouse natural killer cell activity by LS 2616, a new immunomodulator. *J. Immunol.* **134,** 3956.

KALLAND T. & FORSBERG J.G. (1981) Natural killer cell activity and tumor susceptibility in female mice treated neonatally with diethylstilbestrol. *Cancer Res.* **41,** 5134.

KALLAND T. & FORSBERG J.G. (1983) 3-methylcholanthrene: transient inhibition of the lytic step of mouse natural killer cells. *J. natl. Cancer Inst.* **71,** 385.

KASAI M., YONEDA T., HABU S., MARUYAMA Y., OKUMARA K. & TOKUNAGA T. (1981) *In vivo* effects of asialo-GM1 antibody on natural killer activity. *Nature (Lond.)* **291,** 334.

KENNETTT (1980) *Monoclonal Antibodies.* Plenum Press, New York.

KLIMPEL G.R., SARZOTTI M, REYES V. & KLIMPEL K. (1985) Characterization of cytotoxic cells generated from *in vitro* cultures of murine bone marrow cells. *Cell. Immunol.* **92,** 1.

KOO G.C., PEPPARD J.R. & MARK W.H. (1984) Natural killer cells generated from bone marrow culture. *J. Immunol.* **132,** 2300.

KUMAR V., BEN-EZRA J., BENNET M. & SONNENFELD G. (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.

LEDBETTER J.A. & HERZENBERG L.A. (1979) Xenogenic monoclonal antibodies to mouse lymphoid differentiation antigens. *Immunol. Rev.* **47,** 63.

LEE J.C., HAPEL A.J. IHLE J.N. (1982) Constitutive production of a unique lymphokine (IL-3) by the WEHI-3 cell line. *J. Immunol.* **128,** 2993.

LOHMAN-MATTHES M.L., DOMZIG W. & RODER J. (1979) Promonocytes have the functional characteristics of natural killer cells. *J. Immunol.* **123,** 1883.

LOMEDICO P.T., GUBLER U., HELLMANN C.P., DUKOVICH M., GIRI J.G., PAN Y.C.E., COLLIER K., SEMIONOW R., CHUA A.E. & MIZEL S.B. (1984) Cloning and expression of murine interleukin-1 cDNA in *Escherichia coli. Nature (Lond.)* **312,** 458.

MALEK T., ROBB R.R. & SHEVACH E. (1983) Identification and initial characterization of a rat monoclonal antibody reactive with the murine Interleukin-2 receptor-ligand complex. *Proc. natl. Acad. Sci. U.S.A.* **80,** 5694.

MARSHAK-ROTHSTEIN A., FINK P., GRIDLEY T., RAULET D.H., BEVAN M.J. & GEFNER M.L. (1979) Properties and applications of monoclonal antibodies directed against determinants of the Thy-1 locus. *J. Immunol.* **122,** 2491.

MINATO N., REID L. & BLOOM B.R. (1981) On the heterogeneity of murine natural killer cells. *J. exp. Med.* **121,** 1819.

RABIN H., HOPKINS R.F., RUSCETTI F.W., NEUBAUER R.H., BROWN R.L. & KAWAKAMI T.G. (1981) Spontaneous release of a factor with properties of T cell growth factor (Interleukin-2) from a continuous line of primate tumor cells. *J. Immunol.* **127,** 852.

ROSENBERG S.A., GRIMM E.A., MCGROGAN M., DOYLE M., KAWASAKI E., KOTHS E. & MARK D.F. (1984) Biological activity of recombinant human Interleukin-2 produced in *Escherichia coli. Science*, **223,** 420.

SEAMAN W.E., BLACKMAN M.A., GINDHART T.D., ROUBINAN J.R., LOEB J.M. & TALAL N. (1978) b-estradiol reduces natural killer cells in the mouse. *J. Immunol.* **121,** 2193.

STUTMAN O.D., PAIGE C. & FIGARELLA E.F. (1979) Natural cytotoxic cells against solid tumors in mice. I. Strain and age distribution and target cell susceptibility. *J. Immunol.* **121,** 1819.

SUZUKI R., HAUDA K., ITOH K. & KUMAGAI K. (1983) Natural killer (NK) cells as responder to Interleukin-2 (IL-2). I. Proliferation and establishment of cloned cells. *J. Immunol.* **130,** 981.

TALMADGE J.E., MEYERS K.M., PRIEUR D.J. & STARKEY J.R. (1980) Role of NK cells in tumor growth and mestasis in beige mice. *Nature (Lond.)*, **284,** 622.

TRINCHIERI G., MATSUMOTO-KOBAYASHI M., CLARK S.C., SEEHRA J., LONDON L. & PERUSSIA B. (1984) Response of resting human peripheral blood natural killer cells to Interleukin-2. *J. exp. Med.* **160,** 1147.

YOUNG Y.P., OKUMURA K. & MOORE M.A.S. (1985) Generation of natural killer cell lines from murine long-term bone marrow cultures. *J. Immunol.* **134,** 1462.