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Bone marrow and peripheral blood natural killer cell activity in lymphomas. Its response to IL-2

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SUMMARY

Natural killer (NK) cytotoxic activity was simultaneously investigated in bone marrow mononuclear cells (BMMC) and peripheral blood lymphocytes (PBL) from nine Hodgkin's disease (HD) and 15 non-Hodgkin lymphoma (NHL) untreated patients. Twenty-five PBL samples and seven bone marrow specimens from healthy individuals were also included as control group (C). NK cell activity was evaluated in basal condition and post-stimulation with human recombinant IL-2 (rIL-2). Data were expressed in K values (number of BMMC or PBL needed to lyse 50% of the target cells). In basal condition, both HD and NHL patients showed a NK cell activity comparable to the C group, both in BMMC (HD, $K=2{\cdot}48\pm1{\cdot}3$; NHL, $K=3{\cdot}8\pm2{\cdot}0$; C, $K=3{\cdot}2\pm0{\cdot}7$) and PBL (HD, $K=2{\cdot}0\pm1{\cdot}0$; NHL, $K=2{\cdot}3\pm1{\cdot}0$; C, $K=2{\cdot}2\pm0{\cdot}2$). Stimulation with rIL-2 induced a significant and comparable enhancement of the NK activity in PBL from HD, NHL and C while the response to rIL-2 of the BMMC in most of the HD and NHL patients was significantly greater than the C group. Responder cells were characterized by negative selection with specific MoAb plus complement as a $CD3^-$, $CD16^+$, $CD56^+$ cytotoxic cell and further confirmed by flow cytometry. We postulate that IL-2 activation of bone marrow NK cell precursors, in addition to enhancing the activity of circulating NK, may be of value for the therapeutic rationale of IL-2 in patients with lymphoma.

Keywords natural killer cells Hodgkin's disease non-Hodgkin's lymphoma IL-2

INTRODUCTION

Natural killer (NK) cells have been proposed as the first line of defence against tumour and virus-infected cells [1]. These cells arise from bone marrow (BM) precursors, independently of thymic influence [2–4] and their maturation and spontaneous cytotoxic activity is dependent on continued exposure to IL-2 [4–6]. It has been shown that most of the NK function is exerted by a lymphocyte subset known as large granular lymphocytes (LGL) [7], which in addition to their cytotoxic activity is able to secrete a variety of cytokines [8–10]. Although *in vitro* evidence suggests that NK activity may have an important regulatory role against malignant lymphoproliferative diseases [11], NK function has been poorly evaluated in patients with these malignancies. Furthermore, the few available reports in the literature have focused on NK activity only in the peripheral blood (PB) compartment [12,13]. To our knowledge, no previous report has simultaneously evaluated NK function in both BM and PB compartments of patients with Hodgkin's disease (HD) and non-Hodgkin lymphoma (NHL).

In the present investigation, untreated patients with HD and NHL were evaluated for NK activity in BM and PB against K562 cell line, both in basal condition and post-stimulation with human recombinant IL-2 (rIL-2).

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MATERIALS AND METHODS

Patients

Twenty-four untreated lymphoma patients (nine HD and 15 NHL) aged 9–65 years were studied. The clinical protocol included clinical and histological parameters. Within the latter, the Rye Conference Nomenclature [14] for HD and the Working Formulation of Non-Hodgkin Lymphomas for Clinical Usage [15] for NHL was used. Staging of the disease was established following the guidelines of the Ann Arbor Conference [16].

Histological subtypes in the nine patients with HD were nodular sclerosis (three) and mixed cellularity (six); NHL patients were classified as low (five), intermediate (seven) and high grades of malignancy (three), according to the prognostic group.

Controls

PB from 25 healthy adult blood-bank donors and BM aspirates from seven surgical patients (admitted for inguinal hernia

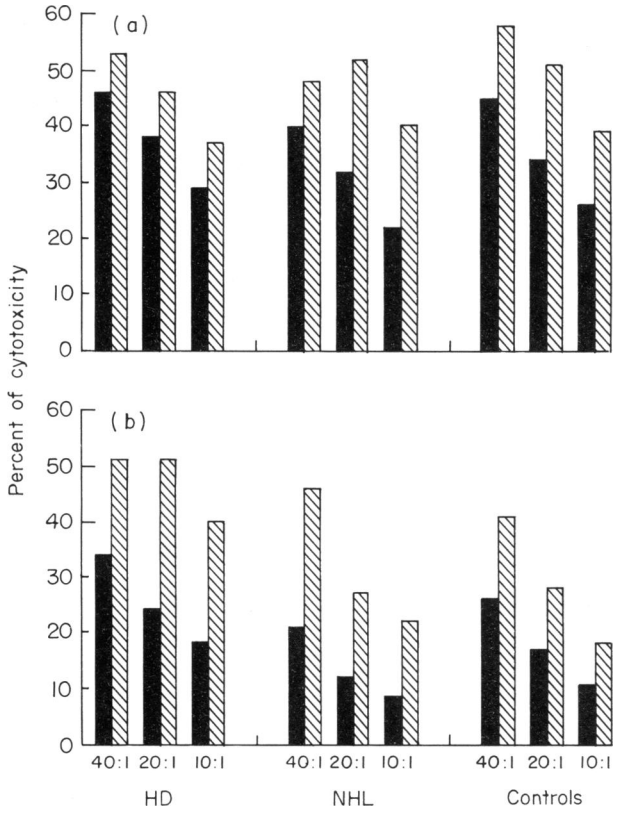

Fig. 1. Comparative NK cell cytotoxic activity from patients with HD and NHL measured in basal conditions (■) and after stimulation with rIL-2 (▨), both in PBMC (a) and BMMC (b).

repair) were included as the control group. Written consent was obtained from each of the seven BM donors.

Samples

BM aspirates were obtained from the posterior iliac crest using standard surgical sterility techniques. Xilocaine 1% was used as local anaesthetic. Aspirates were withdrawn via a Rosenthal needle into a syringe containing 10 U/ml sodium heparin.

Peripheral blood

Fifty milliliters of heparinized blood (10 IU/ml sodium heparin) were also obtained from each individual for PB analysis. Total cell counts were done with a Coulter Counter (Coulter Electronics, Hialeach, FL). Differential counts of 200 cells were also performed on Wright–Giemsa stained smears.

Cell preparation

PB and BM mononuclear cells (PBMC, BMMC) were isolated from heparinized samples by centrifugation on Ficoll-Hypaque gradients as previously described [17]. Both PBMC and BMMC were resuspended in RPMI 1640 medium, supplemented with 10% fetal calf serum (FCS; Gibco, Grand Island, NY), 4 mM L-glutamine, 200 IU/ml penicillin and 100 μg/ml streptomycin. Non-adherent cells from PBMC and BMMC were obtained after depletion of adherent cells (mostly monocytes) by incubation on plastic Petri dishes for 30 min at 37°C in an humidified atmosphere of 5% CO_2–air mixture. In some experiments, the non-adherent cells were further depleted of T cells or NK cells by treatment with anti-CD3 or NKH-1A (CD56) plus complement, respectively.

Cell phenotypes

Cell surface markers were determined by indirect [18] and direct staining of cell suspensions with MoAb (Coulter Corporation, Miami, FL) against CD19(B4), CD3(T3), CD4(T4), CD8(T8), CD56(NKH-1, NKH-1A), CD16 (Leu 11c; Becton Dickinson) 3G8 [19] and were analysed by fluorescence microscopy or in a EPICS 753 (Coulter Corporation, Miami, FL) flow Cytometer. NK cells were also evaluated by morphological characteristics using Cytospin preparations and Giemsa staining [7].

NK Cell cytotoxicity

The human erythroleukaemic cell line K562 [20] was used as the NK sensitive target cell. Five million cells were labelled with 150 μCi of sodium cromate (^{51}Cr, specific activity: 250–500 μCi/mM. Amersham International, Amersham, England) for 1 h at 37°C. Labelled cells were washed three times with RPMI medium plus 5% FCS and resuspended at 5×10^4 cells/ml in RPMI-1640 medium containing 10% FCS. In addition, lymphokine-activated killer (LAK) cell activity was measured against long-term cultured chronic B leukemic cells.

NK activity of non-adherent PBMC and BMMC was evaluated by a ^{51}Cr-release assay as previously described [21]. Briefly, 5×10^3 ^{51}Cr-labelled target cells in 0·1 ml were mixed with 0·1 ml of effector cells (PBMC or BMMC) at three different effector/target cell ratios (40:1, 20:1 and 10:1). Each combination was seeded in triplicate in U-bottomed microtest plates (Falcon Plastics, USA). Triplicates of target cells K562 plus medium were included to measure the spontaneous release. Plates were incubated for 4 h at 37°C in a humidified atmosphere of 5% CO_2–air mixture. Finally, the cells were pelleted by centrifugation at 400 *g* for 10 min and 0·1 ml of the supernatant (per well) was harvested and ^{51}Cr release was measured in a gamma counter (LKB Compugamma, Wallac, Sweden). Total release was determined by counting 5×10^3 lysed K562 labelled cells. The percent of specific lysis was calculated by the following formula:

$$\% \text{ specific lysis} = \frac{\text{mean experimental release (ct/min)} - \text{mean spontaneous release (ct/min)}}{\text{mean total release (ct/min)} - \text{mean spontaneous release (ct/min)}} \times 100$$

The data were also expressed as the number of mononuclear cells required for 50% specific ^{51}Cr release from 5×10^3 K562 target cells (K), calculated by the Trinchieri-modified Von Krogh equation [22] as previously described [23].

IL-2 stimulation

Two million PBMC or BMMC in 1 ml volume were incubated for 18 h at 37°C with 50 U/ml of rIl-2 (Cetus Corp. Emeryville, CA); the cells were washed two times and resuspended at 2×10^6 cells/ml in RPMI plus 10% FCS and subsequently used as effector cells in the NK assays.

Statistical analysis

Results were evaluated for statistical significance between the groups by the Student's *t*-test for non-paired data and between stimulated and non-stimulated data in the same group by Student's *t*-test for matched pairs.

RESULTS

Cell surface phenotype of effector cells

The effector cells in PB and BM from patients and controls were first phenotypically analysed with a panel of six MoAb as described in Materials and Methods. Absolute number of PB lymphocyte subsets from HD, NHL and controls are shown in Table 1. The most consistent finding at this level was a significant reduction of total lymphocytes and of the CD3 lymphocyte subset linked to a decreased CD4 subpopulation. Non-significant alteration was observed on B or NK cells. At the BM level, the composition of the lymphocyte subsets was comparable to the controls (Table 2).

Assessment of lytic capacity

In order to compare the lytic capacity between patients and controls, we have calculated the *K* value for each cell preparation. Furthermore, because NK donors tend to be fairly constant in the rank order of their NK activity, we also express our data as relative *K* (*RK*) to the mean *K* value of a panel of normal fresh controls done at the same time. The application of this method to patient populations makes the comparison of results between patients and controls simpler, since the average value for normal activity is by definition 1·0 [24].

Peripheral blood NK activity

As shown in Table 3, non-significant differences were observed in the basal PBMC NK activity among the three groups (HD, $K = 2{\cdot}0 \pm 1{\cdot}0 \times 10^5$ cells; NHL, $K = 2{\cdot}3 \pm 1{\cdot}0 \times 10^5$ cells; C, $K = 2{\cdot}2 \pm 0{\cdot}2 \times 10^5$ cells). The *RK* also indicated that individual NK activity of HD and NHL patients was similar or lower ($RK < 10$) than the mean control value. Furthermore, only two out of nine HD patients and three out of 15 NHL showed low NK activity as indicated by a $RK > 1{\cdot}0$.

The rIL-2 stimulation induced a significant enhancement of PBMC NK activity in the majority of the patients and in all the controls (Fig. 1). Only three out of eight in HD and two out of 11 NHL were not responders to rIL-2. No correlation was found between the stage or degree of the lymphoma and the response to rIL-2.

Bone marrow NK activity

Table 4 shows the *K* values and its corresponding *RK* obtained from six HD, 12 NHL patients and seven control individuals, all tested for NK activity under basal condition and after adding rIL-2. As determined by *K* values and the *RK*, the basal NK activity in the five HD patients was comparable to or higher than the mean control activity. In NHL, 50% of these patients also showed a NK activity higher than the mean control value. The RK of rIL-2-stimulated BMMC indicated that 60% of HD

Table 1. Phenotypically defined lymphocyte subsets in PBL

	Absolute number of lymphocytes (mean ± s.e.m.)		
Markers	HD ($n=9$)	NHL ($n=15$)	Controls ($n=25$)
Total	1622 ± 181*	1·922 ± 686	2·159 ± 92
CD3	1055 ± 119*	1·610 ± 225	1·451 ± 108
CD4	681 ± 124*	1·155 ± 180	1·010 ± 46
CD8	423 ± 62	647 ± 158	540 ± 27
CD19	294 ± 46	395 ± 50	284 ± 39
CD16	290 ± 36	262 ± 46	332 ± 21
CD56	312 ± 25	295 ± 37	305 ± 23

* Significantly lower than the control value ($P < 0{\cdot}005$).

Table 2. Phenotypically defined lymphocyte subsets in BMMC

	Percent of positive cells ± s.d.		
Markers	HD ($n=6$)	NHL ($n=12$)	Controls ($n=7$)
CD3	36 ± 8	34 ± 7	29 ± 7
CD4	24 ± 6	21 ± 5	24 ± 3
CD8	18 ± 3	15 ± 4	14 ± 2
CD19	13 ± 5	12 ± 3	16 ± 3
CD16	10 ± 2	10 ± 2	9 ± 3

Table 3. NK cytotoxic activity in PBMC

		K values			
		No. of PBMC × 10^5		*RK* values	
Group	Patient No.	Basal	rIL-2	Basal	rIL-2
HD	1	3·0	2·3	1·4	1·1
	2	1·9	0·8	0·9	0·6
	3	2·0	0·03	0·9	0·02
	4	2·1	1·9	1·0	1·5
	5	2·6	2·4	1·2	1·9
	6	0·4	0·002	0·2	0·002
	7	3·1	3·6	1·4	2·8
	8	0·5	0·3	0·2	0·2
	9	2·3	0·6	1·1	0·5
Mean ± s.d.		2·0 ± 1·0	1·3 ± 1·3	0·9 ± 0·5	1·1 ± 1·0
NHL	1	1·7	0·4	0·8	0·3
	2	1·0	0·4	0·8	0·3
	3	2·1	1·5	1·0	1·2
	4	2·5	1·2	1·1	1·0
	5	1·3	1·2	0·6	1·0
	6	2·8	2·1	1·3	1·6
	7	3·7	3·4	1·7	2·6
	8	4·0	3·4	1·8	2·6
	9	1·3	1·0	0·6	0·8
	10	3·5	2·6	1·6	2·0
	11	1·6	0·7	0·7	0·5
	12	2·3	0·6	1·1	0·5
	13	1·8	1·0	0·8	0·8
	14	3·2	2·1	1·5	1·6
	15	1·4	0·7	0·6	0·5
Mean ± s.d.		2·3 ± 1·0	1·5 ± 1·0	1·1 ± 0·4	1·2 ± 0·8
Control (25) Mean ± s.d.		2·2 ± 0·2	1·3 ± 0·3	1·0 ± 0·1	1·0 ± 0·1

K = Cell number needed to cause 50% of target-cell lysis.

Table 4. NK cytotoxic activity in BMMC

Group	Patient No.	*K* values × 10^5		*RK* values	
		Basal	rIL-2	Basal	IL-2
HD	3	3·5	1·6	1·1	0·7
	5	3·5	1·5	1·0	1·0
	6	0·3	0·2	0·1	0·1
	7	3·6	3·5	1·1	1·5
	8	1·9	0·9	0·6	0·4
	9	2·1	0·7	0·9	0·3
Mean		2·48±1·3	1·4±1·2	0·8±0·4	0·7±0·5
NHL	4	3·1	2·5	1·1	1·0
	5	2·8	2·2	0·9	0·8
	6	3·5	2·6	1·0	1·1
	7	7·2	2·0	2·3	0·8
	8	2·2	1·2	0·7	1·3
	9	1·5	0·6	0·5	2·4
	10	2·8	1·3	0·9	0·5
	11	8·4	3·2	2·6	1·3
	12	3·4	2·9	1·1	1·2
	13	2·9	1·0	0·9	0·4
	14	5·4	3·2	1·7	1·3
	15	2·6	0·6	0·8	0·3
Mean		3·8±2·0	1·9+0·9	1·2±0·7	1·0±0·6
Control ($n=7$)		3·2±0·7	2·4±0·5	1·0±0·1	1·0±0·1

K = Cell number needed to cause 50% of target-cell lysis.

Table 5. NK cell activity on PBMC and BMMC after depletion of $CD3^+$ or $CD56^+$ lymphocyte subsets

	Patients ($n=5$)		Controls ($n=5$)	
	Basal	IL-2*	Basal	IL-2
PBMC	40+6	72+6	46+3	58+5
CD3-depleted	ND	61+7	ND	49+2
CD56-depleted	ND	5·2+1·1	ND	2·5+2
BMMC	22+7	50+14	28+7	35+3
CD3-depleted	ND	80+10	ND	31+6
CD56-depleted	ND	8+5	ND	−1·2+3

PBMC or BMMC were selectively depleted of $CD3^+$ or $CD56^+$ subsets by lysing with Anti-CD3 or IgM NKH-1 A (anti-CD56 Coulter) plus complement respectively. Remaining cells were adjusted to 1×10^6 cells/ml and stimulated with 50 u/ml of rIL-2. Cytotoxic activity was tested against K562 at the E/T cell ratio of 40:1. Data are expressed as the mean ± s.d. percent of cytotoxic activity.

and 38% of NHL patients were significantly high responders when compared to the control BMMC.

Effect of the depletion of CD3 or CD56 lymphocyte subsets on the cytotoxic activity

In order to characterize the rIL-2 activated effector cells in both PBMC and BMMC, samples from five patients (one HD and four NHL) and five controls were subjected to NK or T cell depletion before stimulation with IL-2. As shown in Table 5, depletion of $CD3^+$ cells before rIL-2 stimulation did not affect the cytolytic activity of PBMC or BMMC. In contrast, depletion of NK cells with NKH-1A (anti-CD56) plus complement totally abolished the cytotoxic activity in both PBMC and BMMC. Although the IL-2 stimulated killer cells were also tested against a long-term cultured B leukaemia cells, resistant to NK cells, LAK activity was not observed during the selected time (18 h) of incubation rIL-2 (data not shown). A longer period of incubation was not possible to evaluate due to the small number of cells recovered after the depletion procedure.

DISCUSSION

In patients with lymphoid malignancies, cell-mediated immunity, particularly related to T cells [25–27], and to macrophage-mediated functions [28] commonly exhibits significant alterations. However, very few studies are available dealing with the peripheral blood NK activity in HD and NHL patients. Furthermore, the available results are conflicting. Thus, while some laboratories reported significantly depressed NK activity in HD and NHL patients [12,13,27], Gupta & Fernandez [29] have reported a normal NK function in HD patients. To our knowledge, no previous studies have simultaneously examined the NK activity in both PB and BM compartments of patients with HD and NHL.

We basically report on findings related to the nature of NK cell activity in PB and BM from untreated HD and NHL patients. Furthermore, we employed the *K* value and *RK* as useful methods to compare NK lytic activity between patients and controls. The application of the *RK* method to patient populations has the advantage of relating to actual lytic activity as well as reducing intra-assay variation in data expression [24]. It also makes the comparison of results between patients and controls simpler, since the average value for a normal NK activity is by definition 1·0. The application of this criterion to our data permitted us to easily compare patients and controls on an individual basis.

Contrary to previous reports [13,27], our results indicate that PBMC NK cell number and lytic activity are comparable to the controls in both HD and NHL patients. These findings are consistent with those by Gupta & Fernandez [29]. Furthermore, in our patients and controls, rIL-2 induced a significant enhancement of NK activity in both PBMC and BMMC. Moreover, a remarkably higher increase in the BM compartment was noted in many of the HD and NHL patients when compared to the C group. This higher response of the patients' BMMC may be due to an elevated number of NK cell precursors being induced to maturation with rIL-2, since the number of mature NK cells in the BM from patients and controls was not significantly different. This hypothesis is supported by studies showing that the generation of high or low NK cell activity is predetermined at BM precursor level [2]; studies on NK regulation have shown that mouse [30] and human NK cells [31] proliferated in response to IL-2 and that NK cell activity is dependent on their continuous exposure to IL-2 [6]. Furthermore, studies in mouse BM cells [2,32,33] stressed that BM harbours immature NK precursors which are induced to maturation with IL-2.

NK activity in our system was completely abolished by pretreatment with anti-CD56 plus complement but not with anti-CD3, indicating that the nature of rIL-2 activated effector cells belongs to the NK lineage ($CD3^-$, $CD16^+$, $CD56^+$ cells). It should be mentioned that we selected 18 h as incubation period to assess NK activation by rIL-2. This period of incubation is too short to generate LAK cells as has been shown by Lotzova & Savary [34]. However, recent studies by Jhaver [34] have shown that lymphokine-activated killer (LAK) cells generated from peripheral blood of NHL patients expressed a comparable level of cytotoxicity to the control group. Furthermore, the patients with advanced disseminated disease displayed a better augmentation under rIL-2 than the patients with localized disease.

In our study, some patients with advanced HD (Table 1 No. 3, 6 and 8) and NHL (No. 1, 6 and 11) also showed a higher response to rIL-2 in the PBMC, using a short-term 18 h incubation, suggesting that the potential response to rIL-2 is maintained or even increased in both types of malignancies.

Very few reports on the application of IL-2 therapy to lymphoma are available [35]. However, both preclinical and clinical data suggest that lymphoid malignancies may be highly susceptible to IL-2 therapy [36–38].

Our results offer additional support to the possibility that IL-2 activation of NK cells from BM precursors, in addition to enhancing the NK circulating cell activity, may be of value for the therapeutic rationale of IL-2 in patients with lymphomas.

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