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Hematopoietic Origin of Human Natural Killer (NK) Cells: Generation from Immature Progenitors

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Abstract
Human natural killer (NK) cells originate from bone marrow, but little is known about NK cell progenitors and ontogeny. We studied the phenotype and functional activity of NK cells derived from highly purified human bone marrow CD34+ cells, which exhibited neither lytic activity nor expression of surface antigens characteristic of NK (CD56) or T (CD3) cells. However, when cultured with hematopoietic growth factors or feeder layers for up to 4 weeks, up to 86% functional CD56+ cells were seen in the absence of mature T cell development. CD56+ cells appeared in all cultures at 2 or 3 weeks, with the largest percentage in those exposed to IL-2. These studies demonstrated that NK cells arise 'in vitro' from immature bone marrow progenitors and also suggest a separate origin and differentiation pathway for NK and T cells.

Introduction

Natural killer (NK) cells are defined by their non-MHC-restricted cytolytic activity against numerous targets [1–3]. They also express recognizable surface markers such as the CD16 (Fc gamma III receptor) and CD56 (N-CAM) antigens [4–6]. It has been suggested that these cells play an important role in the initial immune response against primary and metastatic tumors [2, 7, 8], viral infections [9], and allografts [10, 11]. They are able to secrete a variety of growth factors and likely influence hematopoiesis, although this has been a subject of controversy [11, 12].

It is well established that NK cell progenitors originate in bone marrow [13–19], and studies in rodent models have already clarified some aspects of their ontogeny and maturation [16–19]. NK cell precursors have been identified in a population of immature bone marrow cells. These cells required, in addition to IL-2 [17], factors produced by adherent (stroma like) cell layers for growth. A murine in vitro long-term bone marrow culture system has been established which allows NK progenitor cell proliferation and differentiation without a need for exogenous cytokines [16, 19]. This culture system does not support the development of mature T or B cells. In fact, the percentage of NK cells has been reported to be much lower when bone marrow cells are cultured under conditions that favor the appearance of T cells [20]. In humans, similar data are currently lacking.

Enriched preparations of human hematopoietic stem cells or progenitor cells, usually prepared by CD34+ cell immunoselection, are increasingly being used in bone

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marrow transplantation [21]. These preparations may allow for ex vivo purging of the contaminating CD34– tumor cells and for expansion of precursors to accelerate myeloid cell engraftment. However, little work to date has focused on reducing the period of immunodeficiency following transplantation. In this regard, it would be useful to know the origin of NK cells and their progenitors and to be able to stimulate their recovery. The present study demonstrates that NK cells can be generated in vitro from highly purified human bone marrow CD34+ cells and that they require IL-2 for their full development and activity.

Materials and Methods

Bone Marrow Progenitors

Bone marrow was harvested from vertebral bodies of normal human cadaveric multiorgan donors as described [22–24]. CD34+ progenitor cells were purified by positive immunomagnetic selection using modified Dynabeads (Dynal, Baxter Corp., Glendale, Calif.) linked via the anti-CD34 monoclonal antibody K6.1 [22–24]. The cells were then cryopreserved in liquid nitrogen vapor phase. In some studies, the cells were cryopreserved for periods of up to 16 months. Immediately prior to culture, they were thawed, washed once in Iscove's modified Dulbecco's medium (IMDM) (Gibco, Grand Island, N.Y.) and counted, and viability was determined by trypan blue exclusion. Viability ranged from 62 to 80%.

Phenotypic Analysis of CD34+ Cells and Effector Cells

Phenotypic studies were performed on CD34+ cells after thawing and after 1–4 weeks of culture by single- and dual-color flow cytometric analysis using either a Becton Dickinson FACScan (Becton Dickinson, Mountain View, Calif.) or a Coulter Epics Elite Flow cytometer (Coulter Electronics, Inc., Hialeah, Fla.). We analyzed the phenotype of cells at day 0 (after thawing) and after 1–4 weeks of culture using the fluorescein (FITC)-conjugated mAb anti-CD45 (Hle-1) -CD3 (Leu-4) and -CD19 (Leu-12) and the phycoerythrin (PE)-conjugated mAb anti-CD3 (Leu-4), -CD16 (Leu-11c) and -CD56 (Leu-19). At day 0, CD34– cells were labeled with an anti-CD34 FITC-conjugated mAb which recognized a different epitope than the one recognized by the antibody K6.1 used for positive selection of the cells. All antibodies were obtained from Becton Dickinson.

After the cells were harvested, they were washed twice and labeled with a saturating concentration of mAb for 15 min at room temperature in the dark. The cells were washed twice in PBS-0.1% sodium azide and then fixed with 1% paraformaldehyde. We found that these cells were >99% CD34+ with a viability ranging from 62 to 80% and did not express significant levels of any of the markers for detecting mature T (CD3), B (CD19) or NK (CD16 and CD56) cells.

Cell Cultures

The CD34– cells were cultured at 37°C in a 5% CO_2 humidified atmosphere for up to 4 weeks in 96-well U-bottom plates (Falcon, Becton Dickinson Labware, Mountain View) at a concentration of 5×10^4 to 10×10^4 cells/ml in 0.2 ml of IMDM supplemented with 10% of fetal bovine serum (FBS) (Hyclone Laboratories, Logan, Utah) and 1% of penicillin G sodium (10,000 U/ml)-streptomycin sulfate (10,000 mg/ml). The different culture systems used included: (a) supplemented media alone; (b) supplemented media containing various recombinant human cytokines in combination, including: interleukin-1-alpha (IL-1-α) (Hoffmann-La Roche, N.J.) 10 U/ml, IL-3 (Genetics Institute, Cambridge, Mass.) 2 ng/ml, stem cell factor (SCF) (Amgen Corp., Thousand Oaks, Calif.) 50 ng/ml, IL-6 (Genetics Institute) 100 U/ml, and granulocyte-macrophage colony-stimulating factor (GM-CSF) (Sandoz, Basel, Switzerland) 200 U/ml; and (c) supplemented media and a feeder layer of irradiated (30 Gy) cells consisting of peripheral blood mononuclear cells (PBMC) isolated by density gradient centrifugation in Ficoll-Hypaque (Histopaque™, Sigma Diagnostics, St. Louis, Mo.).

At the end of the 1st, 2nd and 3rd weeks of culture, 100 U/ml of rIL-2 (a gift from Dr. B. Mukherji) was added to half of the cultures in the same plates. All cultures were continued for another week with or without other cytokines or feeder layers, so that half of the cultured cells were exposed to IL-2 for only 1 week.

The cultures were refed every 3 or 4 days by replacing 0.1 ml of their media with fresh media.

At the end of the 1st, 2nd, 3rd and 4th weeks of culture, cells were harvested and phenotypic and functional analysis were performed. All the experiments were done in triplicate.

Target Cells

The NK-sensitive erythroleukemia cell line K562 was used as a target for cytotoxicity assays. Prior to each assay, viability determined by trypan blue exclusion ranged from 78 to 98%.

Cell-Mediated Microcytotoxicity Assay

Both after thawing (day 0 of culture) and at weekly intervals, when a sufficient number of cells accumulated in culture, a standard ^{51}Cr-release assay [25] was used to evaluate the presence of cytolytic cells derived from CD34+ progenitors. 1×10^6 to 2×10^6 target cells were washed and incubated for 90 min at 37°C with $Na_2{}^{51}CrO_2$ (Dupont, Boston, Mass.) at 0.1 mCi/1 $\times 10^6$ target cells. The cells were then washed five times in IMDM supplemented with 10% FBS and counted. Effector cells harvested from the cultures at the end of the 1st, 2nd, 3rd and 4th weeks were washed and seeded in V-shape microwell plates (Nunc, Naperville, Ill.) at different effector-target ratios (30:1 to 1.87:1) in a volume of 0.1 ml. Labeled target cells at a concentration of 1,000 in 0.1 ml of supplemented media were added. The plates were centrifuged at 150 *g* for 3 min and incubated for 4 h at 37°C in a 5% CO_2 humidified air atmosphere. The plates were then centrifuged at 450 *g* for 5 min; 0.1 ml of the supernatants was removed from each well and withdrawn into aliquots of 1 ml of Aquasol (Dupont). Radioactivity was measured in a scintillation counter (Packard Instrument Company, Downers Grove, Ill.) and the percentage of specific lysis determined by the formula:

$$\text{specific lysis (\%)} = \frac{\text{experimental } ^{51}Cr \text{ release} - \text{spontaneous } ^{51}Cr \text{ release}}{\text{maximum } ^{51}Cr \text{ release} - \text{spontaneous } ^{51}Cr \text{ release}} \times 100.$$

Maximum ^{51}Cr release was determined by adding 0.1 ml of 1% sodium dodecyl sulfate solution (Sigma) to labeled target cells. Spontaneous ^{51}Cr release in 10 experiments, as determined by adding supplemented medium to target cells, averaged 17.5%.

Results

Proliferation of Cells in Culture

CD34+ cells were cultured for 1–4 weeks in media alone or with combinations of either IL-1-α, IL-3, IL-6, GM-CSF and SCF (experiment 1) or IL-1-α, IL-3 and SCF (experiments 2 and 3) in liquid suspension, or on a feeder layer of 50,000–100,000 irradiated peripheral blood mononuclear cells. After the 1st, 2nd and 3rd weeks, rIL-2 was added to half of the cultures, and they were continued for 1 additional week. As seen in figure 1, significant proliferation occurred in cultures in the presence of growth factors. The addition of IL-2 at various intervals did not significantly change the cell numbers when compared with cultures without IL-2. Cells cultured on feeder layers proliferated only in the presence of exogenously added growth factors. Proliferation indexes for the 4-week culture period ranged between 0 and 0.62 when IL-1, SCF and IL-3 were not present in this system (without significant differences between cultures with and without IL-2) and increased to 6.6–24 when these cytokines were added to the cultures over feeder layers.

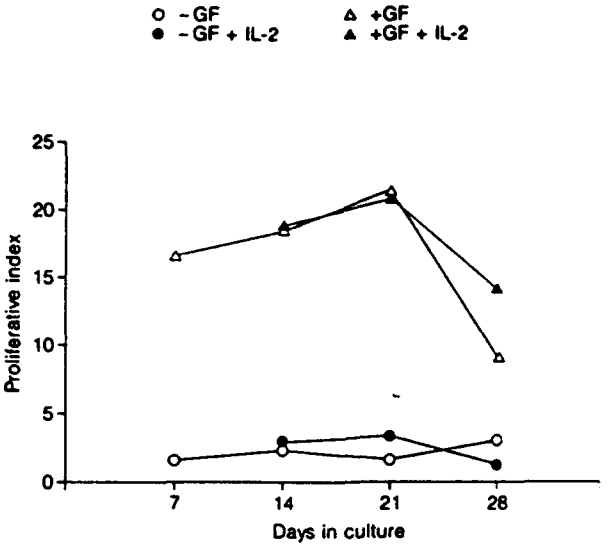


Fig. 1. CD34+ cells were cultured in media alone or with IL-1-α, SCF, and IL-3 (experiments 2 and 3) in a liquid culture system. After week 1, IL-2 was added to half of the cultures, and cell numbers were counted on days 7, 14, 21. Proliferative index was calculated as the number of cells at the different time points divided by the number of cells plated on day 0.

Phenotypic Analysis of Cultured Cells

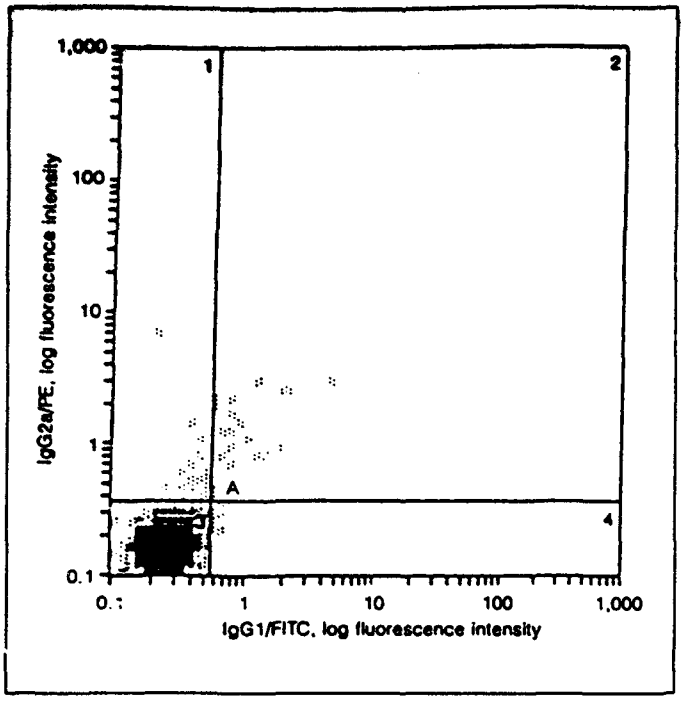
Phenotypic analysis was performed by flow cytometry on day 0 (after thawing) and after 7, 14, 21 and 28 days of culture. Figure 2 shows a dual-color flow cytometric analysis of the cell population before culture, using anti-CD34 FITC-conjugated and anti-CD56 PE-conjugated mAb. Of the CD34+ population, only 0.2% was also CD56+ on day 0. CD56+ cells began to appear at the end of the 2nd week of culture in cells cultured without and with growth factors. The comparison between CD56+ cell numbers in cultures with and without IL-2 clearly demonstrates that the presence of IL-2 in cultures for 1 week was necessary to achieve high numbers of NK (CD56+) cells. Although there was considerable variability among cultures, the number of these cells seemed to peak at the 2nd week for cells cultured with growth factors and the 3rd week for cells grown without factors. As can be observed in tables 1 and 2, mature T cells (CD3+) were not generated in significant numbers in our culture system.

High percentages of CD56+ cells also appeared among cells cultured with feeder layers of irradiated PBMC. As shown by flow cytometric analysis, up to 86% of cells at the end of 3 weeks in these cultures with IL-2 were CD56+ (fig. 3). As an average, CD56+ cells also peaked at day 21. CD56 was detected in only 0–2% of the feeder layer cells irradiated and cultured alone, so we believe that the CD56+ cells seen in the marrow cultures containing feeder layers were generated from the CD34+ cells.

In cultures without exogenous factors or with feeder layers, we could detect small numbers of CD56+ cells at weeks 2 and 3 in the absence of added IL-2. This is different from the cultures to which the exogenous hematopoietic growth factors IL-1-α, IL-3, IL-6, GM-CSF and SCF (but not IL-2) were added. This could be explained by an inhibitory effect of one or more of the growth factors on NK cell development, which could be overcome by the presence of IL-2. To test this hypothesis, we cultured CD34+ cells with IL-1-α, IL-3 and SCF (experiment 4) or IL-1-α, and SCF (experiment 5) for 1 week. We then changed the culture medium to IMDM with FBS with or without 100 U/ml of IL-2. CD56+ cells could not be detected for the following 3 weeks in cultures without IL-2 in both experiments. In contrast, with IL-2, 6–43% of CD56+ cells were detected after 2 and 3 weeks of culture.

Functional Activity of Cultured Cells

Cytotoxic activity of the cultured cells against the NK-sensitive cell line K562 was tested at days 14, 21 and 28.



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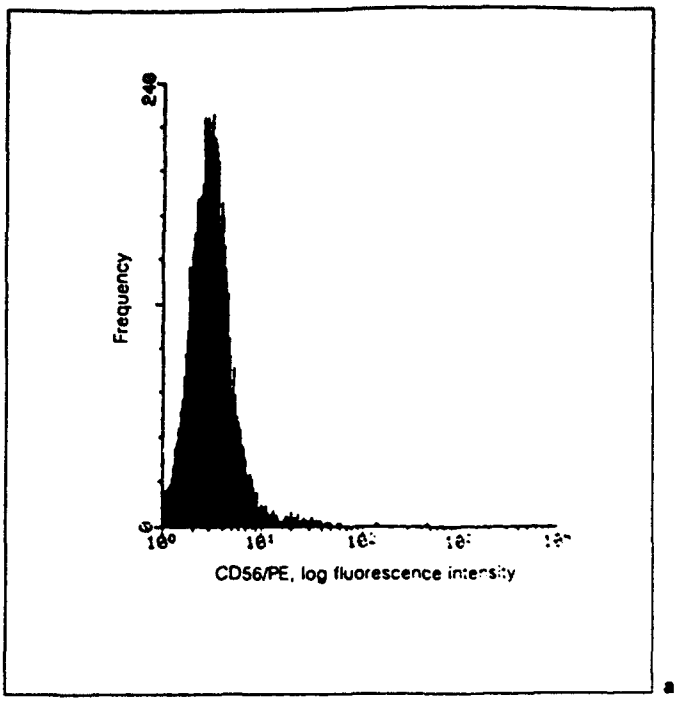

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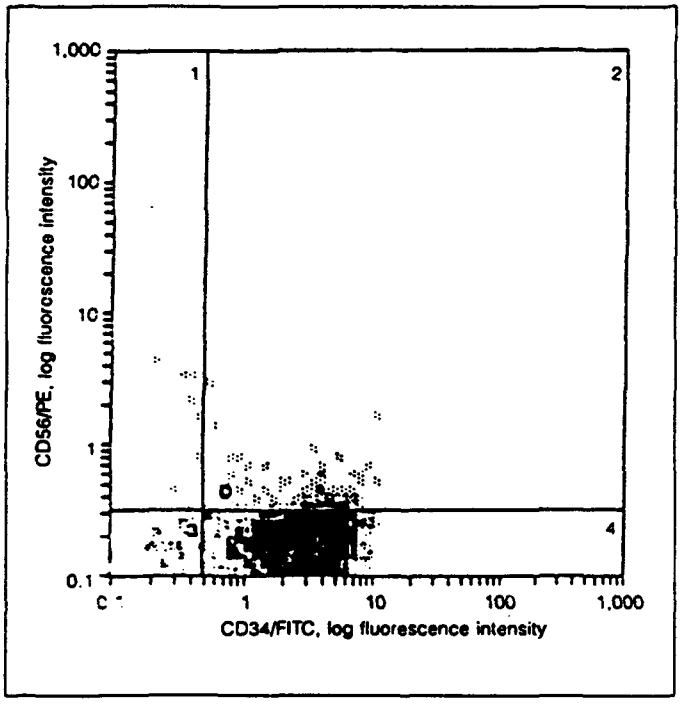

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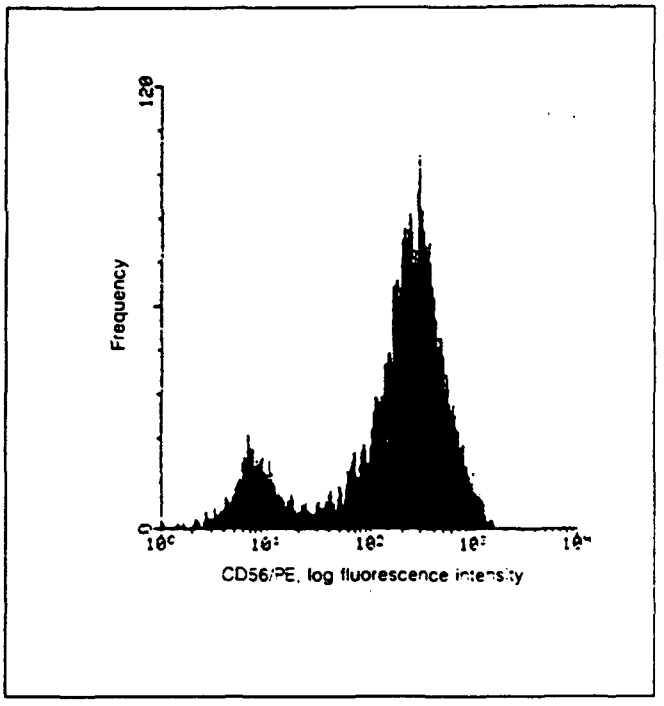

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Fig. 2. Two-color flow cytometric analysis of thawed CD34+ cells at initiation of culture. **a** The log intensity of green (x axis) versus red (y axis) fluorescence of CD34+ cells labeled with irrelevant isotypic antibodies conjugated with FITC and PE (negative control). **b** The log intensity of green (x axis) versus red (y axis) fluorescence of the same cells labeled with FITC-anti-CD34 and PE-anti-CD56 antibodies.

Fig. 3. Flow cytometric analysis for expression of CD56 on CD34+ cells analyzed on day 0 (**a**) or after 3 weeks of culture on a feeder layer of PBMC, containing IL-2 during the last week (**b**). Both show log intensity of red fluorescence (anti-CD56/PE) (x axis) versus number of cells expressing different fluorescence intensity (y axis).

Table 1. Phenotypes of cells without factors

	Day 7	Day 14		Day 21		Day 28	
		-IL2	+IL2	-IL2	+IL2	-IL2	+IL2
CD45							
Experiment 1	ND	100	97.5	74.6	70.0	ND	ND
2	99.2	94.3	98.3	89.6	99.5	96.6	97.8
3	88.3	82.7	94.8	97.8	98.7	81.8	ND
CD56							
Experiment 1	1.5	6.1	15.3	ND	ND	ND	ND
2	3.6	2.3	24.2	4.6	83.5	3.4	55.1
3	1.00	2.1	5.1	4.8	12.3	0.5	ND
CD3							
Experiment 1	0.1	2.3	4.2	ND	ND	ND	ND
2	ND	5.0	1.5	0.4	0.8	0.2	0.9
3	3.00	4.5	2.6	2.5	2.6	2.1	ND

Values expressed as percentage of positive cells. ND = Not done.

Table 2. Phenotypes of cells with factors

	Day 7	Day 14		Day 21		Day 28	
		-IL2	+IL2	-IL2	+IL2	-IL2	+IL2
CD45							
Experiment 1[a]	99.4	93.2	99.4	ND	ND	ND	ND
2[b]	97.8	98.4	96.4	96.8	98.7	99.5	99.1
3[b]	94.6	85.5	94.7	97.7	99.6	96.2	99.6
CD56							
Experiment 1	0.5	0.3	2.0	ND	ND	ND	ND
2	1.3	0.3	15.1	0.9	8.0	3.8	2.9
3	3.2	0.2	21.0	0.5	1.9	2.0	0.2
CD3							
Experiment 1	0.2	3.2	0.0	ND	ND	ND	ND
2	0.2	2.4	0.0	0.4	0.3	0.4	0.3
3	1.7	1.7	4.7	0.4	1.0	2.7	0.6

Values are percentages of positive cells.

[a] Cultures contained IL-1-α, IL-3, IL-6, GM-CSF and SCF.

[b] Cultures contained IL-1-α, IL-3 and SCF.

The results are depicted in figure 4a–c which represent the average for experiments 2 and 3 (where the cells were cultured with IL-1-α, IL-3 and SCF). This shows that cytotoxic activity can only be detected among cells cultured in the presence of IL-2. The functional activity of NK cells in culture appeared to peak at the end of 4 weeks of culture, whereas CD56+ cells appeared 1–2 weeks earlier (tables 1 and 2). It should be noted that the cells cultured with or without IL-2 were washed before the assays so that this cytokine was not present during the assay.

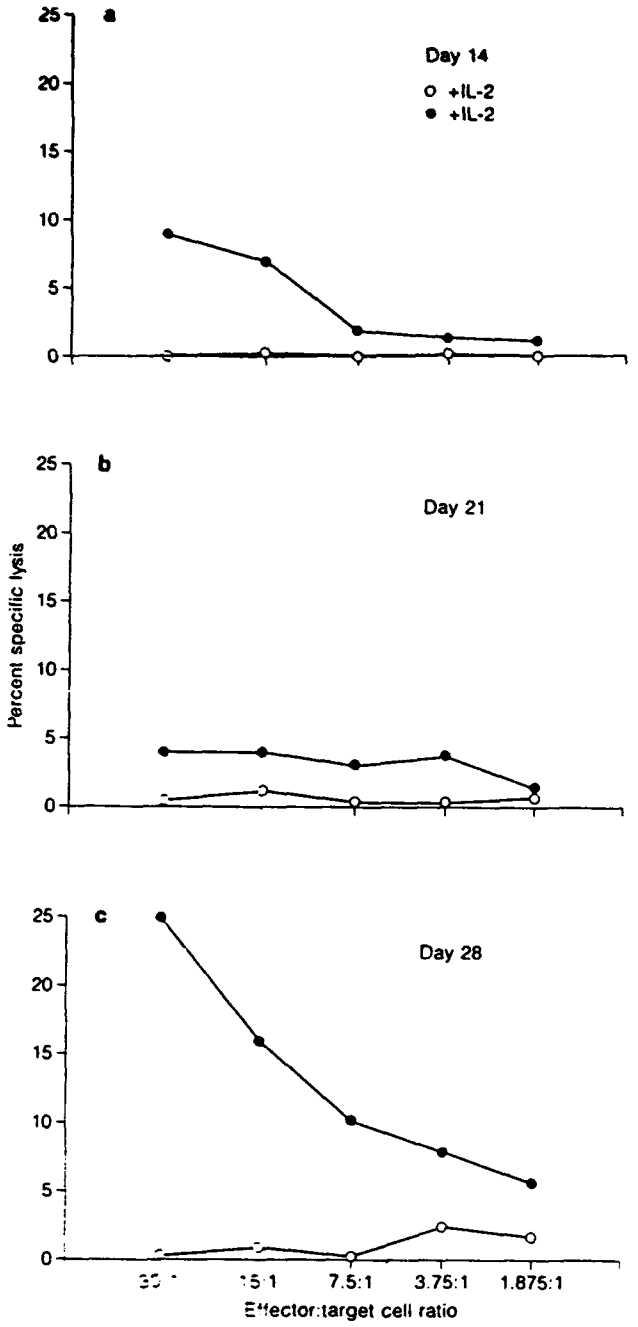

Fig. 4a–c. Cytotoxicity of CD34-derived NK cells, as measured against the K562 cell line, on days 14, 21 and 28. The percentage of specific lysis was calculated as described in Materials and Methods. All experiments were done in triplicate and this represents the average of 2 separate experiments.

Discussion

We studied the origin of NK cells from CD34 positive bone marrow cells. It is well established that human bone marrow contains hematopoietic progenitor cells capable of self-renewal and differentiation into multiple lineages [26, 27]. Recently it has been shown that bone marrow cells bearing the CD34 surface antigen and lacking other lineage-associated markers (CD33, CD15, CD71, CD10, CD5 or CD7) possess these properties and are likely to be very early progenitors able to generate myeloid-, erythroid- and megakaryocytic-committed precursors [26–31]. In vitro proliferation and differentiation of these hematopoietic progenitors require interactions with cytokines, which can be provided by either bone marrow stroma [28, 29] or in solution. Some of the factors known to act on these early progenitors include IL-1 [32, 33], IL-3 [34–36], IL-6 [36] and SCF.

We have established that NK cells, like cells from other hematopoietic lineages, can be generated in vitro from CD34+ purified bone marrow cells. The enriched CD34+ population we used had no cytotoxic activity and was essentially devoid of mature T (CD3+) and NK (CD56+) cells prior to culture: 1.2% of T (CD3+) and 0.2% of NK (CD56+) cells were seen. We realize that this small number of mature NK cells might have expanded during culture; however, this consideration also applies to the clinical setting where patients are transplanted with bone marrow progenitor populations with comparable degrees of purity. The appearance of NK cells after 3 weeks of culture is not likely to be exclusively due to an expansion of mature cells, as has been in other systems [37]. When we diluted human peripheral blood in order to obtain less than 1% of CD56+ cells and cultured it with IL-1, SCF and IL-3 for periods of 2–4 weeks, we could not obtain NK cell development even after IL-2 stimulation for 1 week; a maximum of 3% of CD56+ cells were seen in these conditions. Recently, we have been able to generate NK cells from bone marrow treated 'in vitro' with 4-hydroperoxycyclophosphamide, which destroys all but the more primitive progenitors [38], and cultured in the presence of human AB serum, irradiated bone marrow stroma and IL-2 [unpubl. data]. This also indicates that NK cells can arise 'in vitro' from immature bone marrow progenitors and points to the essential role of IL-2 in this generation.

In our culture system, those cells proliferated only in the presence of hemopoietins added to the culture medium, but the factors were not necessary for the phenotypic expression of NK cell markers. We were unable to

determine if any of the cytokines used in these studies were essential for a preferential development of NK cells from CD34+ cells.

Although there was some variation in the different experiments, the appearance of NK cells was evident in all culture conditions used (with and without growth factors and/or a feeder layer of irradiated PBMN cells) and occurred between the 2nd and 3rd week of culture. In the experiments maintained for 4 weeks, the numbers of CD56+ cells decreased towards the end of the culture period, except in those containing a feeder layer. In some of the experiments, we could detect the appearance of NK cells as early as the end of the 2nd week of culture.

In the presence of growth factors we usually found lower percentages of CD56+ cells than in cultures without cytokines or with feeder layers (see tables 1 and 2): this can be explained by a dilutional effect (the cells were able to differentiate into other lineages) or by an inhibitory effect of one of the cytokines on NK cell development. When IL-6 and GM-CSF were omitted from the culture medium (experiments 2 and 3), we continued to detect CD56+ cells in the cultures with other hemopoietins and rIL-2. An inhibitory effect of IL-3 on early human NK cell development has already been postulated [33, 39]; however, we detected no difference in CD56+ numbers in one experiment when we removed IL-3 from the culture conditions.

Feeder layers of irradiated cells are a known requirement for the cloning and development of T cells. T cells can also produce IL-2, which is an important factor for NK cell proliferation and activation [40, 41]. In our culture systems, we could not obtain significant mature T cell development, as evaluated by the appearance of CD3+ cells. Although no known stimulus for T cell development (such as thymic epithelium or cytokines like IL-7) were present, the lack of emergence of T cells allows us to hypothesize, in agreement with other investigators [20, 42], that NK and T cells have different developmental pathways. This does not exclude the possibility of a common progenitor giving rise to one or the other lineage depending on the microenvironment and cytokines present.

We could only evaluate functional activity of cells cultured with growth factors (with or without feeder layer) after at least 2 weeks of culture. We detected significant cytolytic activity (up to 50%) against the K562 cell line in cells exposed to IL-2 for 1 week, but not in cells grown without IL-2. This was an expected result since NK activity is known to be enhanced by previous exposure to IL-2 [40].

The sequential appearance of NK cell phenotype, IL-2 responsiveness and functional activity has been postulated by Pollack et al. [19] in animal models. Our present results do not allow us to establish such a sequence in humans, since we detected NK cell phenotype and lytic activity at the same time point (2 weeks of culture) in cells cultured with IL-2. However, it should be noted that no markers are available for human NK cell precursors. Functional activity of NK cells seems to be optimum at the end of the 4th week of culture in our experiments, although the highest numbers of CD56 cells were seen at the 2nd and 3rd weeks. Thus the CD56+ cells seen in the early cultures may have been functionally immature.

IL-2 seems to be essential for the full development of human NK cells from their precursors, as it is for mature NK cell activation and proliferation [40, 41]. We could only obtain high numbers of CD56+ cells (over 25%) and cytotoxic activity in cultures containing IL-2 for 1 week. Higher concentrations (1,000 U/ml) or rIL-2 appear to have similar effects [25]. It should also be noted that the short, 1-week treatment of cultures with IL-2 argues against the expansion of a small population of mature CD56+ cells present from the beginning of the culture.

Our results are in contrast with recently published data by Shibuya et al. [43] who characterized NK cell progenitors as being CD34–CD33–CD25+. Different methodologies used to isolate bone marrow progenitors (depletion of monocytes and mature T and NK cells from bone marrow mononuclear cells as opposed to positive selection using immunomagnetic beads) and the fact that these authors only identified NK progenitors after a 24-hour culture with IL-2 may be responsible for these differences. In fact, the presence of IL-2 for 24 h could have induced the loss of markers like CD34 and CD33 while these immature cells were being committed to the NK lineage. The progenitors we used were also CD25+ in a large percentage. They were maintained in cultures in the absence of IL-2 for at least 1 week, and it is possible that during this time they lost the most primitive markers (CD34 and CD33).

In summary, we have been able to demonstrate that functional NK cells, bearing the CD56 surface antigen, can be generated in vitro from CD34+ bone marrow progenitors and that they need IL-2 for their full expression. These conditions did not provide for a significant development of mature T cells. Recently, Savary and Lotzova [44] and Miller et al. [45] were able to generate CD56+ cells with cytotoxic activity from CD34+ enriched bone marrow cells, using stroma cells as feeders. The culture system described here differs from those in that NK cells appear also in cultures supplemented with exogenous

growth factors and without stromata; the presence of a stromal layer does not appear to be essential. A short-term exposure to IL-2 (1 week, as opposed to the 5-week exposure in Miller's culture system [45]) seems to be sufficient to stimulate NK generation. Our studies indicate that, if NK cell immunotherapy is used in the setting of transplantation with early hemopoietic progenitors, a lag time may be needed for the full expression of immunocompetent cells, and IL-2 and other cytokines are essential for full expression of NK cell activity.

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