Natural Killer Cell Precursors in the CD44$^{neg/dim}$ T-Cell Receptor^{neg} Population of Mouse Bone Marrow

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Natural killer (NK) cells develop from the nonadherent cell component of NK long-term bone marrow (BM) cultures (NK-LTBMC). Because these nonadherent cells are depleted of mature NK cells and T cells, but appear to be enriched for NK precursors, they were used as a starting population to begin to define the NK precursors that function in NK-LTBMC. As the stromal cell component of NK-LTBMC has been shown to support interleukin (IL)-2–induced, CD44 dependent, NK cell development from nonadherent NK precursors, NK-LTBMC stroma was used in a limiting dilution assay (LDA) to quantitate the precursors. NK-LTBMC in 96-well plates were irradiated (20 Gy) to kill hematopoietic cells (including the NK precursors), seeded with limiting dilutions of the cells to be quantitated, cultured with 500 U/mL IL-2 for 13 days and assayed for development of NK activity by adding ^{51}Cr-labeled YAC-1 cells to the wells and evaluating the release of ^{51}Cr after 4 hours. Flow cytometric analysis, sorting, and quantitation of the nonadherent cell component of NK-LTBMC showed that NK precursors were concentrated in the CD44$^{neg/dim}$ subset that comprised 10% of the "lymphoid" gated cells. When the CD44$^{neg/dim}$ subset was sorted from BM of mice treated with 5-fluorouracil (5-FU) 1 day before (−1FUBM), there were about 30% T cells, but no NK-1.1$^+$ cells. When the T cells were removed by sorting and the CD44$^{neg/dim}$, $\alpha\beta$, $\gamma\delta$ T-cell receptor^{neg} (TCR$^-$) subpopulation was seeded onto irradiated stroma with IL-2, they proliferated, developed NK activity, became NK-1.1$^+$ and CD44^{bright} and remained $\alpha\beta$, $\gamma\delta$ TCR$^-$. The frequency of NK precursors in this population as estimated from the LDA was about 1/500.

THE STUDY OF natural killer (NK) cell development has lagged behind studies of most other hematopoietic lineages, partly because of a lack of quantitative assays specific for NK precursors.[1] Assays in which colonies develop from lineage committed precursors after culture of whole bone marrow (BM) in semisolid medium in the presence of lineage specific combinations of cytokines have greatly facilitated the study of development of hematopoietic lineages other than the NK lineage.[2] At present, specific combinations of cytokines sufficiently specific to exclusively support colony formation from NK precursors in whole BM are not known. To gain a better understanding of NK cell development, our goal was to develop a clonal assay for NK cell precursors and use it to begin to identify BM subpopulations that include NK precursors that can be quantitated.

Because markers exclusive to NK precursors are unknown, studies of the NK lineage depend on measurements of the function of their progeny, ie, mature NK cells. It is known that complete development from NK precursor to functional NK cell occurs in the BM[3] and that, for early stage precursors, intact BM stroma is necessary for maturation both in vivo and in vitro.[4-6] Furthermore, it is known that cytokines and growth factors, such as interleukin (IL)-1, tumor necrosis factor (TNF)α, and interferon (IFN)γ,[7-10] are involved in NK development, and that the cytokine IL-2 plays a central role in proliferation of NK precursors and their differentiation to mature NK cells,[11-13] although mice lacking IL-2 have NK cells.[14-15] As IL-2 also induces proliferation and activation of mature NK cells, as well as T cells, and both of these cell types can mediate cytolysis of YAC-1 target cells, assay of NK precursors requires separation of the NK precursors from mature NK and T cells.

A NK-long term BM culture (NK-LTBMC) has been characterized that allows the study of the mechanisms involved in the differentiation of NK cells from precursors of both rat and mouse BM.[16,17] The mouse NK-LTBMC has several characteristics that make it useful for initiating and testing procedures for isolation of a functionally pure population of NK precursors and development of a clonal assay. In NK-LTBMC, BM is cultured for 4 weeks without a change of medium and in this interval, the cellularity decreases to 1% to 2% of the number of BM cells initially plated. At this time (day 0), the cultures consist of a nonconfluent layer of adherent stromal cells with small, birefringent, nonadherent cells (loosely attached or floating in the medium). At the day 0 time point, no mature NK cells are detected by either flow cytometry with anti-NK−1.1, by assay of cytotoxicity against the NK-sensitive YAC-1 target cells, or by appearance of cells with large granular lymphocyte (LGL) morphology. In some cultures, T cells are present at a low frequency (<5%). The conclusion that NK precursors are present is based on the 3-day delay after addition of IL-2, before there is cell expansion and an increase in NK1.1$^+$ cells with lytic activity against YAC-1 and LGL morphology, which then eventually represent the majority of the cells in the culture.[17] This development of NK cells is stroma-dependent and requires the activity of the adhesion molecule, CD44.[18]

In the study reported here, we have taken advantage of the characteristics of the NK-LTBMC to develop a stroma-dependent limiting dilution assay (LDA) for quantitation of single NK precursors that proliferate and differentiate to produce cells with NK activity. Studies with the nonadherent cell compartment of NK-LTBMC suggested that the NK precursors were concentrated in the CD44$^{neg/dim}$ subpopulation, but the occasional, small number of T cells in this

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population led us to question the accuracy of their quantitation. However, based on these observations, we proceeded to isolate and quantitate NK precursors from BM of mice given 5-fluorouracil (5-FU) 1 day before harvest (−1FUBM). The BM subpopulation isolated was characterized as $CD44^{neg/dim}$, NK-1.1$^-$, $\alpha\beta/\gamma\delta$ TCR$^-$ and represents less than 1% of the total BM. Within this $CD44^{neg/dim}$, NK-1.1$^-$, $\alpha\beta/\gamma\delta$ TCR$^-$ BM subpopulation, the frequency of NK precursors was estimated to be about 1/500. Although this population is heterogeneous, the culture conditions were specific for development of mature NK-1.1$^+$ cells. The quantitative assays developed, and NK precursor-enriched BM subpopulation identified, should be useful in further defining the cytokines and growth factors that influence NK precursor numbers, the phenotype of NK precursors, and their relationships with other lineages.

MATERIALS AND METHODS

Animals. C57BL/6J mice were purchased from The Jackson Laboratories (Bar Harbor, ME) and used as BM cell donors at 2 to 6 months of age. Mice were housed in an isolated, but conventional colony, and fed Laboratory Chow and acidified (pH 2.4) water ad libitum. The colony has endemic mouse hepatitis virus.

Culture medium. The culture medium (cm) was RPMI-1640 (GIBCO, Grand Island, NY), supplemented with 2 mmol/L L-glutamine, 50 U/mL penicillin, and 50 mg/mL streptomycin. For NK-LTBMC, 5% fetal calf serum (FCS) (Hyclone, Logan, UT) plus 5×10^{-5} mol/L 2-mercaptoethanol was added.

BM cell harvest and NK-LTBMC. BM was obtained from femurs and tibias removed from mice that were killed by cervical dislocation and disinfected by immersion in 70% ethanol. After the bones were removed and cleaned of skin and muscle, BM was exposed by cutting the ends of the bones and expelled by inserting a needle and forcing medium through the bone shaft. A single cell suspension was generated by gentle pipetting, and an aliquot of the cell suspension was counted with a hemacytometer. Aliquots of 2.5×10^7 cells were diluted and plated in T25 flasks (Falcon 3047; Becton Dickinson, Lincoln Park, NJ) in 10 mL of CM. The cultures were then incubated at 37°C with 5% CO_2 in humidified air for 4 weeks without media change.

LDA. An aliquot of 2.5×10^5 cells/well in 0.1 mL of medium was plated in each of the 60 innermost wells of round bottom 96-well plates (Falcon 3077), and the remaining external wells were filled with sterile water. Plates were cultured at 37°C, 5% CO_2 without media change. After 4 weeks (day 0), the plates were exposed to 20 Gy to kill the hematopoietic cells (including NK precursors), while sparing a sparse layer of viable and functional stromal cells. The cells to be tested were seeded at various concentrations onto the stroma, and IL-2 (500 U/mL) was added. For each experiment, seven dilutions were done with 18 to 24 wells/dilution unless otherwise stated. After 13 days, 500 ^{51}Cr-labeled YAC-1 cells were added to the wells and, after an additional 4 hours of incubation, the ^{51}Cr released in 0.1 mL of supernatant was measured. The wells were scored as positive when the cpm were 3 standard deviations (SD) above the cpm of wells with irradiated stroma to which only IL-2 (but no cells) was added at day 0. The frequency of cells generating effectors lytic for YAC-1 (lytic precursor frequency [LPF]) was calculated by a computer program based on a Poisson distribution.[17]

Target cells. The YAC-1 tumor cell line was grown in RPMI 1640 with 10% FCS and subcultured 2 to 3 times/week. For ^{51}Cr labeling the YAC-1 cells were incubated for 1 hour at 37°C with 100 μCi of the radioactive isotope. The target cells were then washed twice, counted, resuspended at a concentration of 1×10^4/mL, and 50 μL were then added to each well.

Reagents. Recombinant human IL-2 (Hoffmann LaRoche, Nutley, NJ) was diluted in medium and used at a concentration of 500 IU/mL. The 5-FU was purchased from Sigma (St Louis, MO) and diluted in 1× Hanks' Balanced Salt Solution (HBSS) (GIBCO, Grand Island, NY) before injecting 150 mg/kg/mouse.

Monoclonal antibodies. The following monoclonal antibodies (MoAbs) were used: biotin-conjugated rat anti-CD44 (IgG2b; clone IM7), fluorescein isothiocyanate (FITC)-conjugated mouse anti-NK-1.1 (IgG2a; clone PK136), phycoerythrin (PE)-conjugated hamster anti-$\alpha\beta$ TCR (IgG; clone H57-597) and anti-$\gamma\delta$ TCR (clone GL3), FITC-conjugated rat anti-B220 (IgG2a; clone RA3-6B2), FITC-conjugated rat anti-CD45 (IgG2b; clone 30F11.1), FITC-conjugated rat anti-CD25 (IgM; clone 7D4), FITC-conjugated anti-Mac−1 (clone 70.11.5), FITC-conjugated anti-Gr−1 (clone RA3-8C5), biotinylated rat anti-Sca−1 (IgG2a; clone E13 161-7), and biotinylated rat anti−c-kit (IgG2b; clone 3C1). For each MoAb, an appropriate isotype control was used. All MoAb and isotype controls were purchased from Pharmingen (San Diego, CA), except rat anti-CD11b (Mac-1; IgG2b clone M1/70.15.1) and rat anti-Gr−1 (IgG2b, clone RB6-8C5), which were obtained from serum-free cultures of the hybridomas, purified, and conjugated with fluorescein.

Flow cytometry. Approximately 1×10^6 cells/sample were pelleted in a round bottom centrifuge tube at 200*g* for 5 minutes. The pellet was resuspended in 10 μL of the predetermined dilution of antibody and incubated on ice for 30 minutes in the dark. The cells were washed twice and resuspended in 1% paraformaldehyde. To reduce nonspecific binding, some samples were incubated on ice for 20 to 30 minutes with mouse serum. The samples were then analyzed using a FACSstar I cytometer (Becton Dickinson, Mountain View, CA).

Isolation of the $CD44^{neg/dim}$ TCR$^-$ population. BM was harvested from mice treated 1 day before with 150 mg/kg of 5-FU. After washing, the sample was depleted of red blood cells (RBC) by incubation for 5 minutes at room temperature with 4 mL of autoclaved RBC lysing buffer (8.32 grams NH_4Cl, 0.84 grams $NaHCO_3$, 0.043 grams EDTA per liter of deionized distilled water). The cells were then washed and resuspended in 0.5 mL of 1× phosphate-buffered saline (PBS) with 10% FCS. The biotin-conjugated anti-CD44 MoAb, PE-conjugated anti-$\alpha\beta$ TCR and anti-$\gamma\delta$ TCR MoAb were added together at predetermined concentrations to the cell suspension. The cells were incubated at 4°C for 30 minutes in the dark, washed twice, and resuspended again for a second step of staining with streptavidin-PE. After two additional washings the cells were filtered through a 40 μm cell strainer (Falcon 2340) and sorted in a FACSstar I cytometer. Populations that fell in the "lymphoid" gate (forward *v* side scatter) and had PE (FL2) negative/dim fluorescence intensity were selected. The lymphoid gate was used because after 4 weeks of culture without medium change or 1 day after 5-FU treatment, we expected the remaining cells to be in Go and, therefore, not in the "blast gate". All sorted populations had ≥98% purity by flow cytometric analysis.

RESULTS

LDA of nonadherent NK-LTBMC cells. Nonadherent NK-LTBMC cells have several characteristics that make them useful for beginning to isolate NK precursor cells. NK precursors are thought to be enriched in the nonadherent population because there is no detectable NK activity, no NK-1.1$^+$ cells (as assayed by one-color flow cytometric analysis), and there are few, if any, T cells.[17] The depletion of NK and T cells was as important as the apparent enrichment of NK precursors in the choice of these cells as a starting population because, in the proposed LDA, all three cell types had the potential of giving a positive response. The impor-

tance of any contribution from mature NK or T cells depended on the relative frequency of NK precursors in the population. Therefore, LDA was done to obtain a first approximation of the frequency of NK precursors plus other responsive cells in nonadherent NK-LTBMC. The LDA result of a cell population that had any mature T or NK cells was described as; the lytic precursor frequency (LPF), to distinguish it from the NK precursor frequency.

To quantitate the LPF in NK-LTBMC, the nonadherent cells from 4-week old NK-LTBMC (day 0) were gently harvested (avoiding detachment of stroma) and counted. They were then replated in LDA as described above. Five experiments were done with seven dilutions each, 18 to 24 wells/dilution for a total of 10 different dilutions for all five experiments. The LPF in the nonadherent component of NK-LTBMC was calculated to be 1/979 ± 231.4 cells. This frequency suggests that "contaminating" mature NK or T cells present at >0.1% might make a significant contribution to the estimated frequency if these cells were as efficient as NK precursors.

Flow cytometric analysis of CD44 antigen on nonadherent NK-LTBMC cells. Because we have previously shown that expression of CD44 was needed for the development of NK cells from precursors in NK-LTBMC,[18] the expression of this adhesion molecule on nonadherent NK-LTBMC cells was analyzed by flow cytometry. As indicated in Fig 1, the anti-CD44 MoAb recognized a majority of cells in the gated population (90%). This distribution is similar to that reported for expression of CD44 on BM cells of several strains of mice.[19]

Sorting CD44 negative/dim and CD44 bright cells from nonadherent NK-LTBMC cells. $CD44^{neg/dim}$ and $CD44^{bright}$ cells were sorted to determine whether or not NK precursors were present or enriched in either of these populations. Sorting was difficult because very few nonadherent cells were available from NK-LTBMC, and they tended to aggregate. After their separation by sorting, the two populations were evaluated for LPF by LDA.[20] Each dilution was assayed in 18 to 96 wells and 12 dilutions were assayed for the $CD44^{neg/dim}$ cells, and 17 dilutions were assayed for the $CD44^{bright}$ cells among four experiments. The linear regression analysis gave a LPF in the $CD44^{neg/dim}$ population of 1/(232 ± 89.4) (r^2 = 0.92) and for $CD44^{bright}$ population of 1/(1370.6 ± 547.6) (r^2 = 0.28). The LPF in the $CD44^{neg/dim}$ population was significantly higher ($P < .025$) than that determined (above) for unsorted nonadherent cell populations of NK-LTBMC (1/(979 ± 231.4) or the LPF of the $CD44^{bright}$ cells and also showed less experimental variability. These data indicate that the $CD44^{neg/dim}$ subpopulation either had more NK precursors or more "contaminating" mature cells than did the $CD44^{bright}$ subpopulation. To distinguish between these possibilities, a more detailed analysis was needed to determine whether or not mature NK or T cells were present in these subpopulations.

Two-color flow cytometric analysis of nonadherent NK-LTBMC cells. Figure 2 shows a two-color flow cytometric analysis of the $CD44^{neg/dim}$ and $CD44^{bright}$ subpopulations. In the upper panel, the scattergram shows that a small number of NK-1.1^+ cells (2.6% ± 1.4%, quadrant 2) was detected in the $CD44^{bright}$ population, but they were present in only

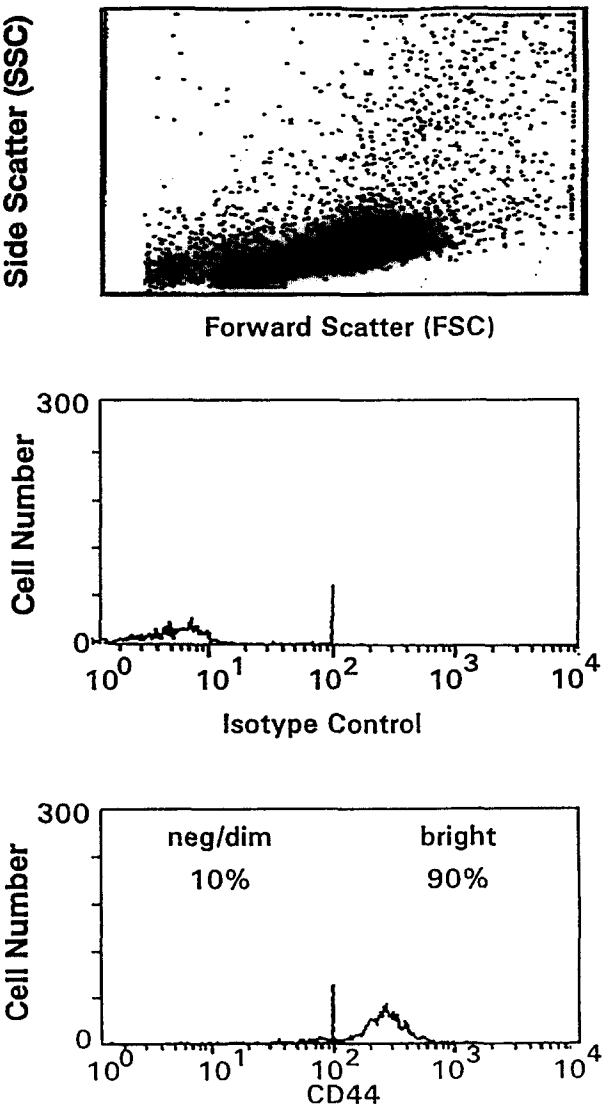

Fig 1. One-color flow cytometric analysis for CD44 expression on nonadherent NK-LTBMC cells. In the upper panel forward scatter versus light scatter is shown. The area outlined is the "lymphoid" gate within which the analysis was performed. The lower panel shows the fluorescence intensity of cells stained for CD44; the vertical line in the histogram represents the gate that was assumed to divide negative/dim and bright cells. The figure is representative of three analyses.

one of three experiments. In the $CD44^{neg/dim}$ compartment, NK-1.1^+ cells were never detected within the limits of flow cytometric analysis, and they always expressed high levels of CD44. As illustrated in the lower panel, $\alpha\beta TCR^+$ cells were consistently present in both the $CD44^{bright}$ (6.6% ± 2.4%, quadrant 2) and the $CD44^{neg/dim}$ (5.5% ± 2.4%, quadrant 1) subpopulations. Based on these data, it seemed likely that the greater experimental variability in the LPF determinations for the $CD44^{bright}$ population was due to the variable presence of NK cells, and that the LPF determined in both populations might be an overestimate of the NK precursor frequency. If the LPF is a function of NK precursors plus T cells, and the T cell frequency is the same for the two

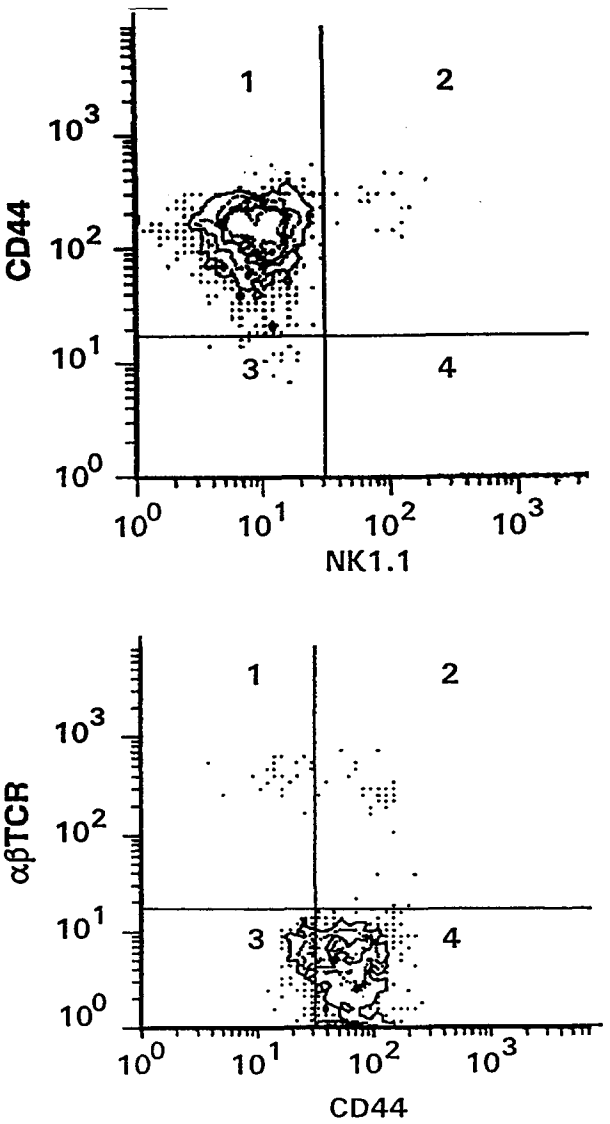

Fig 2. Two-color flow cytometry on nonadherent NK-LTBMC cells. The upper panel shows PE-CD44 versus FITC-NK-1.1, and the lower panel shows PE-αβ TCR versus FITC-CD44. The figure is representative of three experiments.

subpopulations, then the CD44$^{neg/dim}$ subpopulation had more NK precursors. It is known that IL-2 plus a cofactor can activate cytotoxic T cells to kill NK sensitive YAC-1 cells, directly or by secretion of cytokines, which in our LDA would not be washed away before adding target cells. To avoid this possibility, it was necessary to remove the T cells. Because of the small number of CD44$^{neg/dim}$ cells in NK-LTBMC (around 3×10^4/flask), sorting of the cells in these cultures for CD44$^{neg/dim}$ TCR$^-$ cells was impractical. However, based on the above results, it seemed likely that the CD44$^{neg/dim}$ subpopulation of BM would also contain NK precursors and if so, the BM should provide a larger number of cells for analysis.

Searching for the NK precursor in the BM. Because 5-FU treatment of mice is known to kill dividing cells[21] and to enrich for NK precursors and deplete mature NK cells,[22,23] mice were treated with 150 mg/kg of 5-FU and 1 day later, −1FUBM was harvested and the CD44$^{neg/dim}$ cells in the "lymphoid" gate isolated by sorting. As in the nonadherent NK-LTBMC, the CD44$^{neg/dim}$ population from −1FUBM represented only about 10% of the total, and 90% of the cells were CD44^{bright}. The CD44$^{neg/dim}$ cells from −1FUBM were tested in LDA and linear regression analysis ($r^2 = 0.86$) was done on the correlation between percent of negative wells and the log number of cells/well from four different experiments. Each dilution was assayed in 18 to 96 wells. The resultant calculated[20] LPF was 1/(633 ± 325.6). However, as shown below, this population included T cells.

Flow cytometric analysis of CD44$^{neg/dim}$ of −1FUBM. To determine whether or not mature NK cells or T cells were present in the CD44$^{neg/dim}$ population from −1FUBM, two-color flow cytometric analysis was performed using anti-CD44 MoAb in combination with anti-NK−1.1, αβ or γδ TCR MoAb. Table 1 shows that, as was in the case of nonadherent NK-LTBMC cells, the BM-derived CD44$^{neg/dim}$ cells were NK-1.1$^-$; however, there were more T cells (30% ± 2.4% αβ TCR$^+$ cells plus 1.3% ± 0.6% γδ TCR$^+$ cells). Analysis of other surface markers showed a high proportion of cells were positive for CD45, B220, Sca-1, and c-kit, and a few were positive for CD11b (Mac-1) or Gr-1.

Depletion of T cells in CD44$^{neg/dim}$ −1FUBM by sorting. To obtain a more enriched population, T cells were removed from the CD44$^{neg/dim}$ population by sorting for αβ and γδ TCR$^-$ cells. The CD44$^{neg/dim}$ TCR$^-$ cells were then plated (10^3/well) on irradiated NK-LTBMC stroma in medium containing IL-2. At 13 days of culture, there was a large increase in cell number to $40 \pm 1 \times 10^3$ (around 40-fold), and a high proportion of the cells generated were CD44^{bright} NK-1.1$^+$ (73.7% ± 3.6%), with very few (about 5%) being αβTCR$^+$ or γδTCR$^+$ cells, as determined by two color flow cytometry (Table 1).

When the CD44$^{neg/dim}$ TCR$^+$ population were plated in the same conditions, they produced even more cells than had the CD44$^{neg/dim}$ TCR$^-$ cells (Table 2). As expected, most of the cells generated were αβ TCR$^+$ (91.2% ± 2.3%) with <2% being NK-1.1$^+$ or γδ TCR$^+$. The cultured T cells were able to kill YAC-1 cells when the YAC-1 cells were added to the wells as in LDA. This might explain why the LPF was higher in the CD44$^{neg/dim}$ subpopulations with T cells than those without T cells. The amount of contribution of T cells, however, may be complex because the antibodies used

Table 1. Flow Cytometry on CD44$^{neg/dim}$ Population of −1FUBM

Surface Markers	Positive Cells (%)
NK1.1	0 ± 0
αβTCR	33.0 ± 2.4
γδTCR	1.3 ± 0.6
CD45	95.3 ± 1.8*
B220	80.1 ± 8.4
Sca-1	69.3 ± 18.5
c-kit	64.5 ± 14.8
Gr-1	11.9 ± 2.3
CD11b (Mac-1)	5.2 ± 0.6

* The numbers represent the mean ±1 SEM from three different experiments.

Table 2. Comparison of TCR⁻ and TCR⁺ Cells in the CD44$^{neg/dim}$ Population After 13 Days of Culture on Irradiated Stroma With 500 IU IL-2

Cell Population	Cell Number* ($\times 10^3$)	Phenotype (%)†		Positive Wells With Cytotoxic Activity‡ (%)
		NK-1.1	$\alpha\beta$ TCR	
TCR⁻	41 ± 1	73.7 ± 3.6	2.5 ± 0.9	28
TCR⁺	345 ± 35	1.1 ± 0.1	91.2 ± 2.3	83

* Number of cells/well recovered 13 days after the addition of cells plus IL-2 to wells with irradiated stroma. The cells from all wells were pooled and counted and the numbers are the mean ±1 SEM of three experiments. In these experiments 1,000 cells/well were plated.

† Mean ±1 SEM for data from three experiments. Cells from all wells were pooled to do flow cytometric analysis of phenotype.

‡ In this experiment, 500 cells/well were plated with IL-2 on irradiated stroma and 13 days later ^{51}Cr-YAC-1 cells were added. Calculation of positive wells was the same as in LDA.

to sort the cells may activate the T cells and make them more responsive in the LDA, and on the other hand, activated T cells may suppress NK precursor function. In view of the aggressive growth of sorted CD44$^{neg/dim}$ TCR$^+$ cells to produce >90% T cells after culture, and the fact that CD44$^{neg/dim}$ TCR$^-$ cells produced predominantly NK1.1$^+$ cells, with only 5% T cells, suggests that either there were no contaminating T cells or they were much less efficient than the sorted positive cells (possibly activated by anti-TCR antibodies).

LDA of CD44$^{neg/dim}$ TCR$^-$ cells from −1FUBM. Because cultures of the CD44$^{neg/dim}$ TCR$^-$ cells were virtually devoid of mature NK cells and T cells by flow cytometry, the LPF in this subpopulation should most accurately reflect the NK precursor frequency. Therefore, CD44$^{neg/dim}$ TCR$^-$ cells were plated in LDA together with IL-2 and the estimated frequency of NK precursors in this subpopulation was 1/(493 ± 198.9; see Fig 3).

Although the flow cytometric analysis of CD44$^{neg/dim}$ cells showed no NK-1.1$^+$ cells, there remains a slim possibility that a very low level of mature NK cells were present. We tested this in two ways. We found that in two of three sorts, there was no NKRP1 (the gene complex that encodes for the NK-1.1 surface molecule) mRNA detectable by reverse transcriptase-polymerase chain reaction (RT-PCR) (data not shown). The sensitivity of the RT-PCR method used was sufficient to detect mRNA from one mature NK cell among 5,000 YAC-1 cells (NKRP1 negative).

Another approach was to test the ability of the isolated CD44$^{neg/dim}$ TCR^{neg} population to proliferate in response to IL-2 in fresh medium (FM) in the absence of stroma. Mature NK cells can proliferate under these conditions, whereas precursors apparently need stroma or at least stromal cell conditioned medium. Two sorts were done and 10^3 sorted cells were plated per well of a 96-well plate with either irradiated stroma or FM. Both groups were given 500 U/mL IL-2. The cell count on day 13 was 29 ± 14 × 10^3 for the cells on stroma and 3 ± 1 × 10^3 for cells in FM. This indicated that for substantial proliferation, the responding cells needed stroma or the cytokines produced by stroma, and thus seem not to have been mature NK cells.

DISCUSSION

Here, for the first time, a BM subpopulation has been identified that contains NK cell precursors that could be quantitated in vitro. As previously reported,[18] the NK precursors were dependent on BM-derived stromal cells and IL-2 for development into mature NK cells. The NK precursors identified appear to represent a later stage of commitment to the NK lineage than the "transplantable NK precursors" (TNKP) studied in other laboratories.[24,25] The phenotype of the TNKP has been studied by treating mice with various antibodies plus complement.[24] Mice treated with anti-QA-2 or anti-H-2 antisera, but not mice treated with anti-NK-2.1, asialo GM$_1$, Qa-5 or Thy-1,[25] were depleted of NK precursors. In studies that were concurrent with ours, TNKP were phenotyped by cell sorting as Ly6$^+$, Lin$^-$, ckit$^+$, CD43^{high}, fall-3^{high}, TSA-1$^-$, AA4.1^{low}, and Rh123^{high}.[25] These markers indicate that the TNKP has the phenotype of a metabolically active (blast gate, Rh123^{high}) lymphohematopoietic stem cell. The TNKP have not been quantitated, and quantitation would be very difficult because the assay requires measurement of NK activity in NK-depleted, lethally irradiated mice injected with the BM subpopulation to be tested. Such repopulation could theoretically come either from a stem cell or a TNKP.

The purpose of the present study was to identify and isolate a mouse BM subpopulation enriched in NK precursors and to quantitate their frequency in vitro. This has been done by using the information gained by studying NK-LTBMC. The NK-LTBMC contain NK precursors that are IL-2 and stroma-dependent, which is an important characteristic of these precursors because it provides a basis for distinguishing the NK precursors from mature NK cells. The stromal cells, necessary for support of NK cell development from precursors, were obtained for the LDA by irradiating established 4-week old NK-LTBMC. It had previously been shown[17] that 20 Gy irradiation of the 4-week NK-LTBMC resulted in effective elimination of the hematopoietic precursors (including the NK precursors), but did not compromise the ability of the stroma to support NK cell development from NK precursors that were seeded onto the irradiated cultures with IL-2. This culture system would allow the study

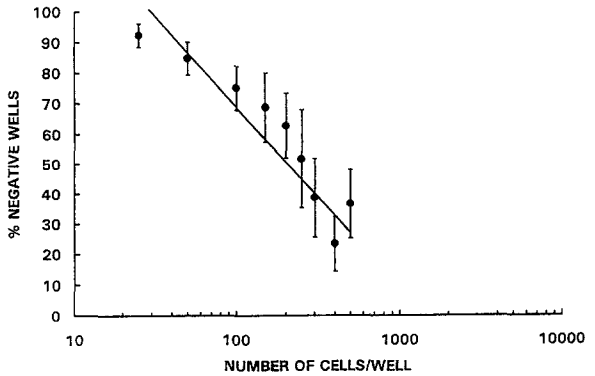

Fig 3. LDA of CD44$^{neg/dim}$ TCR$^-$ cells from −1FUBM. Linear regression (r^2 = 0.96) of the correlation between percent of negative wells and the log number of cells/well from three different experiments. Each point is the mean ± 1 SEM of 36 to 72 wells.

of NK precursors in any cell population lacking mature NK and T cells. As BM is the physiologic site of NK development in the adult mouse, BM is, therefore, an important source for study.

Previous studies of the NK-LTBMC also suggested that the nonadherent cell population from NK-LTBMC would be a good starting population in which to quantitate NK precursors, and the previous finding that CD44 function was important to NK development in these cultures[18] suggested a marker that might be useful. $CD44^{bright}$ cells constituted about 90% of the cells on both NK-LTBMC and −1FUBM and in whole BM from several strains of mice.[19] Furthermore, all of the $NK1.1^+$ cells were $CD44^{bright}$. Even with the presence of occasional $NK1.1^+$ cells and about 7% T cells, the $CD44^{bright}$ nonadherent subpopulation of NK-LTBMC had a lower LPF than did the total nonadherent population or the $CD44^{neg/dim}$ subpopulation, which had no flow cytometric detectable $NK1.1^+$ cells and about 6% T cells. This suggested that the NK precursors were concentrated in the $CD44^{neg/dim}$ subpopulation. This was somewhat unexpected in view of the necessity of CD44 for their proliferation and differentiation in NK-LTBMC and suggests that increased expression of CD44 may be a feature of maturation of NK precursors. Our system should enable further study of the role of this widely distributed molecule that expresses many splice variants[26] and has also been shown to be important for differentiation of myeloid and B cells.[27]

Step-wise isolation and assay of BM subpopulations, using MoAbs to surface antigens, is a strategy that has previously been used to obtain subpopulations highly enriched for hematopoietic stem cells.[28,29] A similar approach is being used for TNKP,[25] and the present studies represent a first step in enrichment of a quantifiable, stromal-dependent NK precursor. It is difficult to calculate the amount of enrichment of NK precursors achieved by isolating the $CD44^{neg/dim}$ TCR^- population. Unlike lymphohematopoietic stem cells or myeloid precursors that can be assayed in whole BM, NK precursors can only be assayed after enrichment. An estimate of the percentage of cells might be made by considering the percentage of cells remaining after each step in the procedure. The −1FUBM cellularity is about one half that of normal BM,[30] (our observations), and is enriched for primitive hematopoietic precursors and stem cells.[31,32] Assuming NK precursors are in each selected population, the lymphoid gate represents about 25% of the −1FUBM, 10% of these are $CD44^{neg/dim}$, and 70% of these are TCR^- so the final precursor-containing subpopulation represented less than 1% of the whole BM and thus could have been enriched as much as 100-fold.

Despite the estimated enrichment, the frequency of NK precursors remained fairly low in the $CD44^{neg/dim}$ TCR^{neg} subpopulation of BM; about 0.2%. The fact that sorted T cells are exposed to anti-CD44 and anti-TCR antibodies before culture with NK-stroma and IL-2 produced a positive read-out in the LDA assay and, though not tested directly, mature NK cells are well known to proliferate and become more active at killing YAC-1 cells after exposure to IL-2, points out the importance of eliminating these cells from the test population. In our studies, effort was made to remove these cells, and several lines of evidence suggest that we succeeded. By flow cytometric analysis no $NK1.1^+$ cells were detected. However, RT-PCR for mRNA of the gene encoding the NK1.1 protein is more sensitive and would detect the mRNA even if the protein was not yet expressed on the surface of the cell. When we devised and used a RT-PCR assay that could detect the mRNA of a single mature NK cell mixed with mRNA from 5,000 negative cells, and the mRNA from three different sorts was tested, only one sort gave positive results. This shows that cells with mRNA for NK1.1 are less than one tenth as common as the estimated NK precursor frequency. This is partly because mature NK cells are $CD44^{bright}$. Further evidence for the presence of stromal-dependent NK precursors was that no significant proliferation or development of cytotoxicity occurred in the absence of stroma, whereas more mature cells would respond to IL-2 without stroma (see, for example, Migliorati et al[22]). The development of 75% $NK1.1^+$ in the cultures provided evidence that only NK precursors were responding. In the 96-well plates, it is difficult not to harvest some stromal cells, which may have been the other 20%, in addition to 5% T cells.

These studies describe a subpopulation of BM cells that contain NK precursors that can be quantitated and a clonal assay system for these precursors. There are several probable levels of NK precursors in BM. The first step beyond the lymphohematopoietic stem cell may be TNKP; then IL-2–nonresponsive, stromal-dependent precursors; followed by IL-2–responsive, stromal-dependent precursors; which are followed by IL-2–responsive, stromal-independent cells and cells that bind, but do not kill target cells. The nature of the assay is likely to determine which precursor is characterized. For example, by definition, the stem cells and TNKP need to be studied in vivo.[24,25,28,29] The IL-2–nonresponsive precursors may be the ones measured in Dexter-like cultures of $CD34^+$ DR^- human cells.[33] Our stroma-dependent LDA assay allows the detection of precursors that are either themselves IL-2–responsive or depend on substances produced by the stroma to respond to IL-2. Similar observations have recently been made with human BM $CD34^+$ subpopulations that have <1% cells with markers for mature T or NK cells.[33-37] One laboratory reported development of NK cells from BM, without the use of a preformed stroma, when IL-2, IL-1α, and stem cell factor are present.[34] Their starting population was 10 to 20 $\times$ 10^3 $CD34^+$ cells per well of a 96-well plate and because the $CD34^+$ population is known to contain stromal cells,[38] these stromal cells could have influenced NK development over the 5-week growth period. Questions as to which precursor(s) is (are) functioning in these various assays and what factors regulate their survival, proliferation, and differentiation can now be addressed with a more defined murine BM subpopulation than was previously available, and such results can be compared with those obtained with subpopulations producing NK cells in human BM or producing NK cells after transplantation into lethally irradiated mice.[25] In both the human and mouse, there is now a limiting dilution assay to study clonal populations derived from a single cell and to determine the frequency of NK precursors in the same or different stages of development.

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