Stromal Cells Derived from Spleen or Bone Marrow Support the Proliferation of Rat Natural Killer Cells in Long-Term Culture

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Abstract. **Rat nylon wool nonadherent bone marrow cells were propagated for up to 75 days in co-culture with stromal cells derived from either spleen or bone marrow. Interleukin (IL) 1 enhanced the ability of spleen stroma to support the long-term culture of natural killer (NK) cells, ostensibly by inducing these support cells to synthesize other cytokines. Flow cytometry studies indicated that the nylon wool separation procedure enriched the concentrations of mature NK cells from 7.9% to 38.1% for splenocytes and from 3.8% to 19.5% for bone marrow cells. Analyses of the adherent zones of suspended nylon screen NK cell cultures revealed substantial numbers of large granular lymphocytes that expressed NK 323^+/MOM/3F12/$F2^-$ phenotypes. The presence of both mature and immature cells of the NK lineage in this matrix was inferred by the presence of both IL-2 receptor (IL-2R) positive and IL-2R negative, and $OX\text{-}8^+$ and $OX\text{-}8^-$ NK 323^+ cells over the >4-month experimental period. Suspended nylon screen cultures displayed a greater potential for producing cytolytic cells than either co-cultures of bone marrow nonadherent cells on stromal monolayers or suspension cultures. The large granular lymphocytes produced in suspended nylon screen cultures could be transformed into active killers of YAC-1 targets by IL-2. In contrast to bone marrow nonadherent cells, more splenic nylon-wool-passed cells displayed a mature NK phenotype, but their proliferative potential and ability to be transformed into cytolytic cells by IL-2 decreased rapidly in culture. In the suspended nylon screen culture system, NK cells migrate from the underlying stroma in stages as they mature, retain their cytolytic potential, and manifest a capacity for self-renewal. Cultured cells were routinely dissociated into single cell suspensions via enzyme treatment and were reinoculated onto "fresh" nylon screen/stromal cell templates after passage through nylon wool columns. These co-cultures continued to generate cytolytic cells in numbers greater than those of the initial inoculum.** [P.S.E.B.M. 1992, Vol 200]

Cytotoxic lymphocytes with broad range antitumor activity can be isolated from peripheral blood or various lymphatic tissues and acquire strong cytolytic activity against a wide variety of fresh and cultured tumor cells when incubated with recombinant interleukin (IL) 2 (rIL-2) (1–3). This activity is not major histocompatibility complex restricted. Natural killer (NK) cells are responsible for most of the lymphokine-activated killer (LAK) activity in humans (4) as well as in rats (5, 6). Although IL-2 can induce the proliferation and activation of LAK cells after short time periods of culture (6), these effector cells cannot be expanded for long terms in culture, and, with time, their cytolytic activity is lost (7, 8). LAK cell therapy may prove useful for the treatment of solid tumor cancers; several groups report significant tumor regression in some patients with advanced cancer treated using this method (9, 10). However, other patients either do not respond to LAK therapy or manifest severe toxicity reactions (9, 10). The former may be attributed to the lack of sufficient numbers of activated

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cells to be therapeutically effective, or the presence of cells such as macrophages (11), certain T lymphocyte subpopulations (12), or granulocytes (13), which have been reported to inhibit NK cell function. The toxicity can almost certainly be ascribed to the use of IL-2 *in vivo* to optimize cytolytic activity.

Bone-marrow-derived hematopoietic cells require the presence of hematopoietic organ stroma or a combination of factors elaborated by these cells in order to be sustained in culture (14). Considerable functional heterogeneity has been reported for bone marrow stromal cells (15, 16) which, when established in monolayer, form microenvironments capable of supporting discrete hematologic lineages. True multilineage hematopoiesis, however, is supported only for 1 to 3 weeks in these systems, and, although active hematopoiesis may continue for months, the cellular products are primarily granulocytes and macrophages (17). Recent work has focused, with some success, on prolonging the active hematopoietic period of this type of culture by adding exogenous growth factors, either singly or in combination (18, 19). The production of these and other regulatory cytokines by stromal cells presumably diminishes with increasing time periods in culture or these support cells may elaborate inhibitory factors. This stromal monolayer-based co-culture method has been adapted for use in several species, including humans (20). In addition, specialized co-cultures were developed that preferentially support the maturation of lymphoid cells (21), whose expression is low in monolayer-type, long-term bone marrow cultures (14, 17). Culture of rat, monkey, and human bone marrow cells on suspended nylon screen/stromal cell templates appears to extend the capacity to support multilineage hematopoiesis over longer terms (22–24), possibly because the three-dimensional matrix engenders more patent interactions between stromal cells and the hematopoietic elements which they support.

The present report describes an adaptation of the suspended nylon screen culture method using IL-1-stimulated splenic stroma to support the growth of rat bone-marrow-derived NK cells for extended periods *in vitro*.

Materials and Methods

Fisher/Copenhagen F1 hybrid rats were obtained from Harlan Sprague-Dawley, Inc.; handling of the rats conformed to the NIH Guidelines for the Care and Use of Animals. Animals were given food and water *ad libitum* and were used at 10 weeks of age or older (≥200g).

Preparation of the Nylon Screen Template. Nylon filtration screens (No. 3-210/36; Tetko, Inc.) were used as templates for stromal cell growth (22–24). These were composed of nylon filaments of 90 μm in diameter that were oriented in a square weave pattern with sieve openings of 210 μm. Pieces (23 mm × 23 mm) of screen were soaked in 1.0 *M* acetic acid for 30 min, washed with distilled water, coated with solubilized Type IV mouse collagen (Gibco) for 1 hour, and incubated in fetal bovine serum (FBS) for 1 to 2 hr to enhance cellular attachment.

Stromal Cell: Bone-Marrow-Derived Nylon Wool Nonadherent Cell Co-Cultures. Endosteal and medullary femoral bone marrow was mechanically disaggregated into single cell suspensions and plated into 25-cm^2 flasks containing 5–7 ml of medium. Splenic cells were procured by forcing 10–20-mm^3 pieces of tissue through an 18-gauge needle into a petri dish filled with medium. Clumps were teased apart with forceps, filtered through a 280-μm nylon screen, and incubated in complete medium (35°C, >90% humidity). After 6–8 hr, nonadherent cells were removed and cryopreserved for future use. Stromal cell expansion was carried out for three to four passes in RPMI 1640 medium conditioned with 20% FBS (Medium A). The screens were placed in six-well plates and inoculated with 2×10^6 splenic stromal cells in 1 ml of this medium. After 3–4 hr of incubation (5% CO_2, 35°C, >90% humidity), the screens were transferred to different six-well plates and gently floated in 5 ml of Medium A. Adherence and growth were monitored microscopically. After 5–8 days, when spleen or bone marrow stromal cells completely surrounded each nylon filament and extended processes across (but did not completely cover) three to four out of every five sieve spaces, the screens were transferred to six-well plates containing 1 ml of modified Mishell-Dutton (25) medium with 2.75% FBS, 2 m*M* glutamine, 1 m*M* sodium pyruvate, 0.1 m*M* nonessential amino acids, 5×10^{-5} 2-mercaptoethanol, and antibiotics in RPMI 1640 medium (Medium B), which was supplemented with 0.5–6 half maximal units of human interleukin 1 (hIL-1) (Collaborative Research). The screens were inoculated 1 hr later with 10^6 bone marrow or spleen cells that did not adhere to a packed nylon wool column (5, 26), incubated for 3 to 4 hr, and transferred to new six-well plates containing Medium B supplemented with hIL-1 for 2 days. Seeding efficiency was determined by comparing the numbers of cells that were unattached 3–6 hr after inoculation to the numbers in the inoculum. This averaged ~45%. Co-cultures were fed every second day with a 10:1 (v/v) solution of Medium B:Medium C (1.37 m*M* essential amino acids, 0.5 m*M* nonessential amino acids, 10 m*M* L-glutamine, 17.85 m*M* $NaHCO_3$, and 1.0% dextrose in RPMI 1640 medium) supplemented with 1 half maximal unit hIL-1/ml. Identical feeding protocols were employed for monolayer cultures of splenic or bone marrow stroma that were inoculated with nylon wool nonadherent cells after ~70% confluence was achieved or nylon wool nonadherent cells that were plated into 25-cm^2 flasks without stromal cells. Cells in

suspended nylon screen co-cultures were concentrated in two main areas; the adherent zone in which hematopoietic cells develop within the stromal matrix and a quasiadherent zone consisting of loosely attached cells found between the nylon screen and the bottom of the culture chamber. These latter cells were removed by gentle flushing with medium at each feeding and cryopreserved. In addition, periodic transfer of hematopoietic cells was performed when the nylon screen co-cultures started to approach confluence. Cells were dissociated enzymatically and single cell suspensions were passed through a packed nylon wool column as described previously; nylon wool nonadherent cells were then reinoculated onto new nylon screen/stromal cell matrices. This step was performed to prevent stromal cells from overgrowing the developing NK cells and inhibiting their proliferation or maturation.

To generate effector cells, cultures were treated with Type I collagenase and layered over a packed nylon wool column. Nonadherent cells were inoculated onto ~70% confluent monolayers of spleen stromal cells in six-well plates containing 5 ml of Medium B supplemented with 1000 IU/ml of rIL-2 (Hoffman-LaRoche). Human interleukins were found to be active on rat stromal and hematopoietic cells in a previous study (2). Stromal cells were employed to enhance the attachment of the quasiadherent cell population. After the initial 24-hr period, cultures were fed daily for 5 to 7 days with Medium C supplemented with 1000 IU/ml of rIL-2 and either evaluated for cytolytic activity or cryopreserved for future study. The methods are schematized in Figure 1.

Evaluation of Cytolytic Activity. Adherent zones of suspended nylon screen or monolayer-based cultures of different ages were treated with collagenase Type I (Sigma Chemical Co., St. Louis, MO) and single cell suspensions were obtained. Quasiadherent cells were removed by gentle flushing with medium and pooled. Intact, suspended screen cultures or cells derived from them were transferred to six-well plates containing modified Mishell-Dutton medium with 1000 units/ml of rIL-2 for 5 to 6 days. The six-well plates used for single cell suspensions contained stromal cell monolayers that were about 70% confluent. Cytolytic activity was quantified using the radiochromium release assay. Briefly, 150 μCi of $Na_2{}^{51}CrO_4$ (New England Nuclear) were incubated with 5×10^6 target cells, washed, and seeded into 96-well microplates at 2×10^4 cells per well. NK-sensitive YAC-1 cells and NK-resistant P-815 cells were used as targets. Suspensions of effector cells were added in triplicate wells at various effector to target cell ratios in a final volume of 200 μl. The samples were centrifuged at 500*g*. After incubation for 4 hr at 37°C, 100 μl of supernatant were collected from each well, and the radioactivity was measured in a gamma scintillation counter (Beckman Instruments) to determine experimental release. Spontaneous release (SR) was obtained from wells receiving target cells and culture medium only and total release (TR) was determined from wells containing target cells and 10% sodium dodecyl sulfate. The percentage of cytotoxic activity was calculated by the following formula:

$$\% \text{ Cytotoxicity} = \frac{(\text{ER}) - (\text{SR})}{(\text{TR}) - (\text{SR})} \times 100$$

Phenotypic Analysis. Analysis for NK cells was performed using the following Serotec monoclonal antibodies: MRC MOM/3F12/F2, which recognizes mature granulocytes (27) and myeloid precursors (28); OX-8, which binds $T_{\text{supressor/cytotoxic}}$ cells and NK cells (29); NK 323, which reacts with NK cells and 80% of the granulocyte population (30); OX-54, against the CD2 epitope (29); and OX-39, which recognizes the IL-2 receptor. To deplete myeloid elements, cells liberated from the adherent zone via enzyme treatment were washed and placed in complete medium for 1 to 2 hr at 36°C and 5% CO_2 and incubated with MOM/3F12/F2 for 1 hr on ice. They were washed and incubated for 2 hr with Dynal magnetic microspheres, which were covalently bound to goat anti-mouse IgG_1. The MOM/3F12/F2$^+$ (myeloid) cells were depleted by circulating the sample through a magnetic aperture using a Harvard peristaltic pump. The cellular effluent (MOM/3F12/F2$^-$ cells) was washed, incubated with OX-8 (1 hr/4°C), washed again, and reacted with goat anti-mouse IgG_1-rhodamine (Cappel Inc.) for 1 hr. After repeated washing, cells were incubated with NK 323-fluorescein isothiocyanate (FITC) and evaluated with an EPICS C flow cytometer (Coulter Electronics) set at a wavelength of 488 nm and appropriately gated to segregate the large granular lymphocytes (LGL) population. MOM/3F12/F2$^-$ cells that reacted with either NK 323 alone or NK 323 and OX-8 were considered to be natural killer cells. Further characterization was carried out using OX-54 and OX-39. The large granular lymphocyte morphology was verified after sorting. Even though most of the myeloid cells become trapped in the nylon wool column and, in any event, do not propagate well under these culture conditions, the magnetic microsphere step was employed as a precautionary measure to eliminate any granulocytes that might be recognized by the NK 323 antibody.

The percentage of NK 323$^+$ cells expressing IL-2 receptors was determined by sorting OX-39-labeled cells with immunomagnetic beads. OX-39$^+$ and OX-39$^-$ cells were then labeled with NK 323-FITC. Control cells were incubated with mouse IgG_1 (Coulter Immunology) followed by goat anti-mouse IgG_1-FITC. Cells labeled with mouse IgG_1-FITC or IgG_1-rhodamine were used as direct controls where appropriate. The following

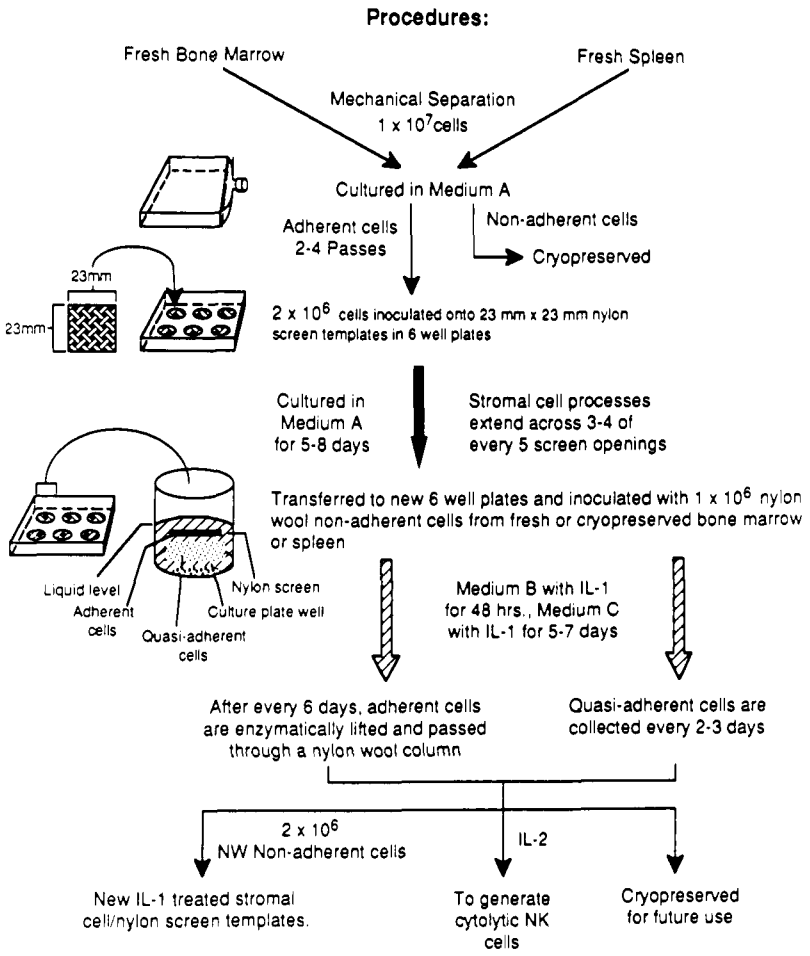

Figure 1. Schematic of the experimental protocol.

additional antibodies were employed for the phenotypic analysis of the packed nylon wool separation procedure for bone marrow cells: MRC W3/25 (against T_4 lymphocytes) (31), MRC ED-1 (reacting with monocytes and macrophages), and MRC OX-33 (recognizing the leukocyte common antigen on rat B cells) (32).

Electron Microscopy. Scanning electron microscopy was performed on pieces of nylon screen cultures, which were subjected to vacuum dessication and coated with colloidal gold using an Edwards Sputter coater. Specimens were viewed with a JEOL T-330 scanning electron microscope.

Statistical Analysis. Means and SE were calculated from a minimum of three cultures for each experimental group. Levels of significance were determined using Student's *t* test. Flow cytometry measurements were made in triplicate on sample sizes of 6,000–10,000 cells.

Results

Stromal cells from either bone marrow or spleen attached to collagen-coated nylon screens, completely enveloped each nylon strand by 4–6 days, and extended processes across three to four out of every five sieve spaces by 5–8 days after inoculation. The nylon wool column procedure positively selects for both spleen and bone marrow NK cells (5, 26); we observed yields of 15% and 40% of the initial cell numbers, respectively. This step eliminated all of the phenotypically mature B cells, most of the monocytic and myeloid cells, and 36% of the OX-8 reactive cells from bone marrow, but enhanced the concentration of NK cells isolated from both bone marrow (from 3.8% to 19.5%) and spleen (from 7.9% to 38.1%) (Table I). A seeding efficiency of 40–45% of these cells onto the stromal cell/nylon screen templates was observed. Active lymphopoiesis associated with stromal cells was evident as early as 3 days after inoculation. Cells removed from suspended screens after 75 days of culture and passed through packed nylon wool columns displayed a morphology that was consistent with LGL at either the light or electron microscope level (Fig. 2, A and B). Scanning electron microscopy studies revealed that LGL cells displayed a multidimensional growth pattern on the suspended nylon screen/stromal template (Fig. 2B). The adherent zones of nylon screen cultures contained

Table I. Phenotypic Analysis of the Separation of Fresh Splenocytes and Bone Marrow Cells on Packed Nylon Wool Columns[a]

Cell source	NK[b]	B[c]	T_4	OX-8	My	Mo
Bone marrow	3.8 ± 0.34	5.78 ± 0.44	3.78 ± 0.44	3.61 ± 0.43	12.97 ± 0.90	9.97 ± 0.78
Spleen	7.9 ± 0.61	12.15 ± 1.14	18.25 ± 2.86	17.51 ± 3.13	—	6.42 ± 0.90
NWP-BM	19.5 ± 1.63	1.40 ± 0.32	3.11 ± 0.98	2.31 ± 0.75	3.48 ± 0.38	2.04 ± 0.53
NWP-S	38.1 ± 2.47	—	—	—	—	—

[a] Effect of the nylon wool column procedure on the distribution of bone-marrow- or spleen-derived cells. NWP, cells passed through a packed nylon wool column; BM, bone marrow; S, splenocytes. Results are expressed as mean ± 1 SE. —, not measured.
[b] Determined as the percentage of cells with large granular lymphocyte morphology (moderate to high forward light scatter characteristics with moderate right angle light scatter properties) that display the NK 323^+/MOM/3F12/$F2^-$ phenotype.
[c] These monoclonal antibodies have the following specificities: B ($OX\text{-}33^+$); T_4 ($W3/25^+$, small to medium sized agranular cells); OX-8 (suppressor/cytotoxic T cells and natural killer cells); My (myeloid cells recognized by MRC MOM/3F12/F2); Mo (monocytic/macrophagic cells reacting with ED-1); OX-54 (CD2); and OX-39 (IL-2 receptor).

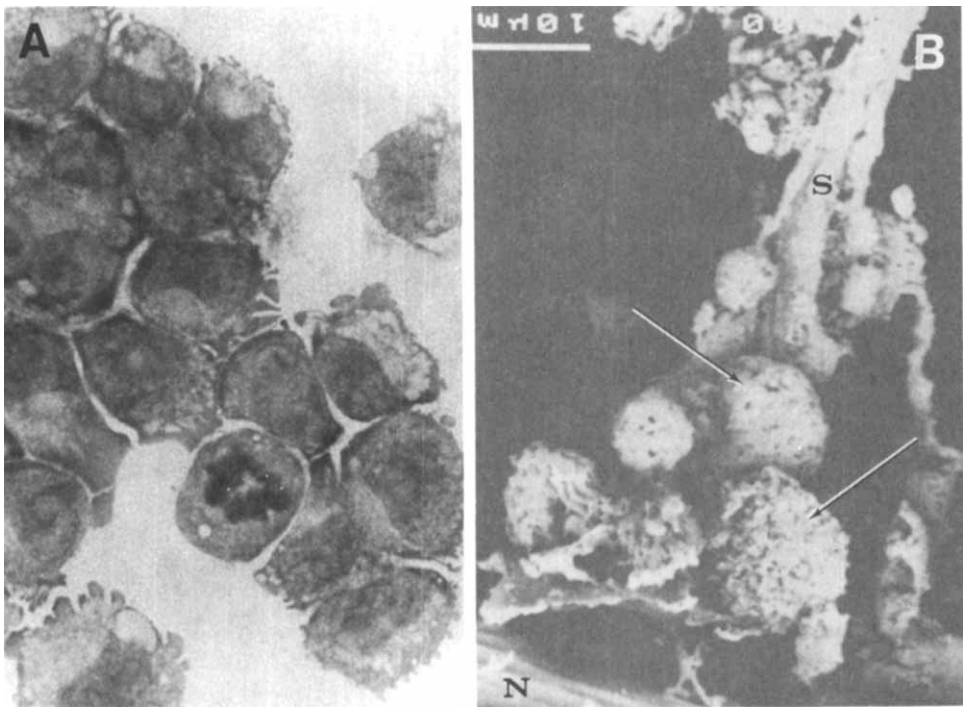

Figure 2. (A) Cytosmear of cells removed from a 75-day suspended nylon screen culture by enzyme treatment and then passed through a nylon wool column. All cells show a typical LGL morphology and a mitotic figure is evident also. (Diff Quik staining; original magnification ×1000). (B) Scanning electron microscopy of a suspended nylon screen culture at 15 days after inoculation showing the association of LGL (arrows) with spleen stroma (S) attached to the nylon filament (N) in the foreground (original magnification ×1200).

substantial numbers of NK cells (NK 323^+/MOM/F12/$F2^-$) for up to 121 days *in vitro* (Table II). In contrast to the NK 323^+ cells of the initial inoculum (8.1%), 43.8–51.0% of nylon-wool-passed cells from cultures >24 days old expressed IL-2 receptors (IL-2R). The NK 323^+/$IL\text{-}2R^-$ cells probably represent a more primitive cell population. A total of 32–45% of the NK 323^+ cells were also $OX\text{-}8^+$ over the same time periods in culture. The expression of CD2 antigen on the adherent zone cells varied between 13.7% for the initial inoculum to a high of 25.8% at 24 days of culture. Depletion of cells that potentially cross-react with the NK 323 antibody (myeloid cells) was accomplished by removing these cells with Dynal magnetic microspheres bound to goat anti-mouse IgG_1 after labeling with MOM/3F12/F2. This antibody binds to epitopes on myeloid precursors (24) as well as mature granulocytes (4).

IL-1, which was used in this system to stimulate proliferation and secretion, also enhanced attachment of the NK cells. In addition, we found that this agent stimulated collagen synthesis. The 1 half maximal unit dose of IL-1 produced optimal NK growth in the co-cultures, inducing a >400% rise in the numbers of these cells in 6-day cultures as compared with cultures not receiving this cytokine ($P < 0.001$) (Fig. 3). In contrast, IL-1 levels lower than a ½ half maximal unit of IL-1

Table II. Phenotypic Analysis of Cells Dissociated from the Adherent Zones of Nylon Screen Cultures of Various Ages[a]

Culture age (days)	NK	OX-8	OX-54	$NK^+/IL\text{-}2R^{+b}$	$NK^+/IL\text{-}2R^-$
0	22.2 ± 1.32	4.0 ± 1.20	13.7 ± 2.05	8.1 ± 2.56	86.4 ± 5.69
15	57.5 ± 3.90	—	—	—	—
24	52.0 ± 4.24	15.4 ± 1.52	25.8 ± 2.07	47.5 ± 2.52	53.7 ± 3.08
49	44.5 ± 3.21	17.7 ± 1.29	22.9 ± 2.41	50.3 ± 2.03	48.6 ± 1.71
75	69.0 ± 6.20	—	—	—	—
105	53.5 ± 3.88	11.6 ± 1.17	15.3 ± 0.95	43.8 ± 4.53	49.9 ± 3.27
121	35.4 ± 2.79	5.6 ± 0.91	17.8 ± 1.24	51.0 ± 3.00	41.8 ± 2.92

[a] Analysis of the adherent zones of nylon screen co-cultures established with bone-marrow-derived nylon wool column nonadherent cells and splenic stromal cells. —, not measured.
[b] The percentage of NK 323^+ cells that were either $IL\text{-}2R^+$ or $IL\text{-}2R^-$ (next column).

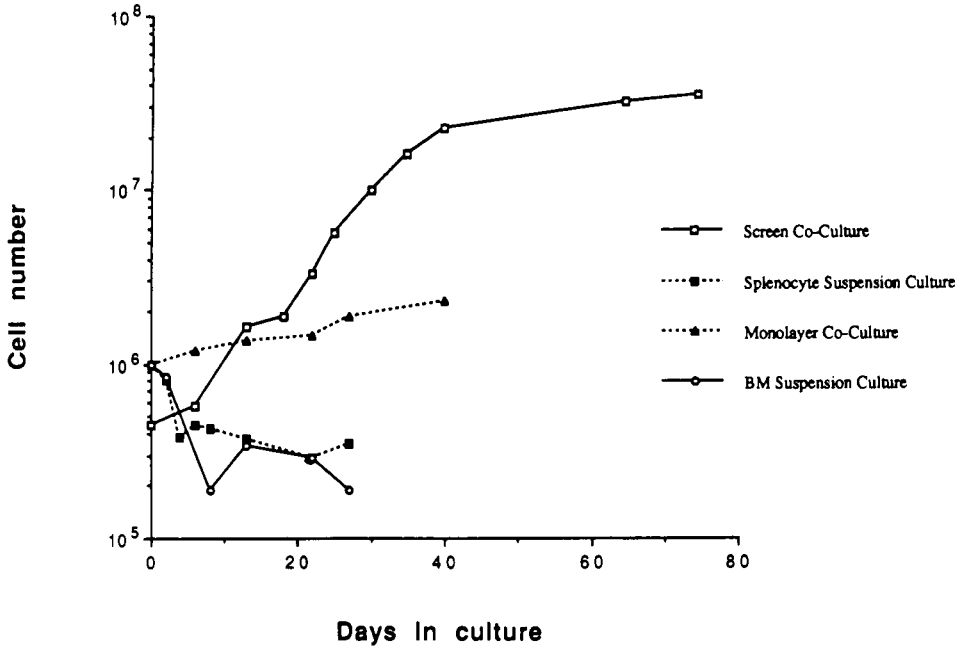

Figure 3. Absolute cell numbers generated by suspension cultures, stromal monolayer-based co-cultures, and suspended nylon screen co-cultures of nylon wool nonadherent cells. Co-cultures consisted of bone-marrow-derived nylon wool nonadherent cells and splenic stromal cells. All cultures were fed with media containing IL-1. The numbers of NK cells in nylon screen cultures were enhanced by IL-1 treatment. Based on an initial seeding of 4.5×10^5 cells, the cultures produced 6.9×10^5 and 7.3×10^5 NK cells after culture in medium without IL-1 for 3 and 6 days, respectively, as compared with 9.7×10^5 and 2.55×10^6 NK cells from cultures treated with 1 half maximal unit of IL-1 for equivalent time periods.

induced neither stromal nor NK cell proliferation; IL-1 doses exceeding 1 half maximal unit promoted excessive collagen synthesis and actually inhibited proliferation (data not shown). IL-1-treated stromal cells also supported the growth of bone marrow-derived NK cells in monolayer conditions, but the proliferation of these cells was more rapid and extensive on suspended nylon screen templates (Fig. 3). Stromal cell-associated lymphopoiesis in either the suspended nylon screen system or the monolayer-based co-cultures was substantially higher and occurred for much longer periods than in suspension cultures of nylon-wool-passed bone marrow cells or splenocytes (Fig. 3). The numbers of cells recovered from suspension cultures steadily diminished with time, with no evidence of self-renewal. We did not observe any differences in the ability of spleen-derived versus bone marrow stroma to support the proliferation of NK cells, and the co-culture data reported herein are accrued from bone-marrow-derived, nylon wool column nonadherent cells supported by splenic stroma.

Constant egress of LGL from the top surface of the screen to the area between the screen and the bottom of the tissue culture flask was observed. rIL-2 can transform these loosely attached quasiadherent cells into lymphocytes with cytolytic properties. These cells can be transferred to fresh stromal cell cultures for further expansion or harvested and cryopreserved for future use. IL-1 alone did not enhance the cytolytic

activity of either the adherent or quasiadherent cells of suspended nylon screen cultures (Table III). In the suspended nylon screen system, bone-marrow-derived NK progenitors retained their proliferative potential for more than 2 months (Fig. 3). In addition, the rate of turnover was rapid, with the cell number doubling every other day. When stimulated with 1000 IU/rIL-2/ml/day, these cells became actively cytolytic against YAC-1 targets (Fig. 4). P-815 killing was low and, evidently, nonspecific (Tables IV and V). The cultures, therefore, apparently do not produce the cytotoxic T cell component of LAK in significant numbers. The nylon wool column isolation procedure, which decreases the non-NK OX-8$^+$ population by greater than one third, may have selected out these cells or they may not proliferate under the culture conditions we employed. The cytolytic activity of the adherent cells was significantly higher ($P < 0.01$) than that of cells derived from the quasiadherent zone (Table IV) harvested just prior to enzyme treatment of the nylon screen. However, the total quasiadherent cell numbers which were collected and stored over time were substantially higher than the numbers found in the adherent zone at the time of digestion. In this regard, the mean ratio of quasiadherent to adherent cell numbers was 6.67:1. No significant differences in the cytolytic activity of NK progenitors supported with bone marrow stroma versus spleen stroma were observed (Table V). Although the cytolytic activity of bone-marrow-derived NK cells diminishes with time in culture, it is maintained at higher levels and for longer periods in the suspended nylon screen co-culture than in either the monolayer-based co-culture or suspension cultures that lack stroma (Fig. 4). In addition, the absolute numbers of effector cells produced in the suspended nylon screen culture were substantially higher than those observed with the other *in vitro* methods (Fig. 3).

Discussion

Bone marrow is the major hematopoietic tissue in adult mammals and is responsible for the production of erythroid and myeloid cells, as well as megakaryocytes and B and null lymphocytes. In addition, stem cells originating in the bone marrow seed various lymphatic organs, including the thymus, where they differentiate to mature T lymphocytes and attain immunological competence. Hematopoiesis occurs in a microenvironment provided by various types of stromal support cells that include reticular cells, macrophages, adipocyte-like cells, various types of fibroblasts, and endothelia (33). These cells provide trophic/differentiation factors to the developing hematopoietic cells, as well as secreting a matrix to which they attach. A similarity exists between organ stroma of all hematopoietic or formerly hematopoietic tissues. Fetal hematopoietic organs, such as the liver and the spleen, can re-exhibit hematopoietic support if bone marrow function is compromised (34). Whereas the capacity to support myelopoiesis is a feature primarily related to certain subpopulations of bone marrow stroma (15), cells with the ability to support erythroid and megakaryocytic growth may also be found in the spleen and liver. In contrast, all lymphoid organs contain stroma capable of supporting various types of lymphopoiesis. In the present study, flow cytometry evaluation indicated that bone marrow contains only about 50% of the mature NK cell population of the spleen. Other groups have reported that NK progenitors and effectors are phenotypically distinct (5) and that bone marrow contains higher numbers of these progenitors than spleen (35). Stromal cells support the growth of NK progenitors for 75 to 120 days in suspended screen cultures. In addition, stromal monolayers can support NK cells for several weeks (Fig. 3) (8, 36), and these cells exhibit cytolytic activity after IL-2 treatment (Table V; Fig. 4). In contrast, the phenotypically mature NK cells isolated from the spleen can be cultured for only short periods and do not show evidence of self-renewal (Fig. 3). The adherent zones of nylon screen cultures contained substantial numbers of NK cells; it is likely that the phenotypic distribution of these cells represents a wide

Table III. Mean Percentage of Cytotoxicity[a] (±1 SE) of Fresh Bone Marrow and Nylon-Wool-Passed Bone Marrow Cells Cultured on Suspended Nylon Screen/Stromal Cell Templates with or without IL-1 Treatment[b]

Target[d]	Effector[c]		
	SPS and NWP-BM	BMS and NWP-BM	Fresh NWP-BM
YAC-1	13.5 ± 2.8 (12.9 ± 1.8)	12.7 ± 3.0 (12.3 ± 2.1)	8.5 ± 2.7 (5.7 ± 1.3)
P-185	4.0 ± 2.3 (3.1 ± 1.7)	4.1 ± 2.2 (2.4 ± 1.2)	2.7 ± 1.5 (2.1 ± 0.8)
Splenocytes[e]	1.5 ± 0.9	1.8 ± 1.0	0.0 ± 0.0

[a] Endogenous activity; no IL-2 was added.
[b] Measurements for cells not receiving IL-1 are in parentheses.
[c] Effector cells were obtained following passage through a packed nylon wool column prior to assay for cytolytic activity. These originated from a freshly prepared bone marrow suspension or from cultures of nylon wool nonadherent cells on nylon screen templates with either spleen stroma (SPS) or bone marrow stroma (BMS). NWP, nylon wool passed.
[d] Target cells were incubated with 150 μCi of $Na_2{}^{51}CrO_4$ per 5×10^6 cells, washed, and seeded into 96-well microplates at 2×10^4 cells per well. The target cell to effector cell ratio was 1:50.
[e] Fresh spleen cells from a normal rat.

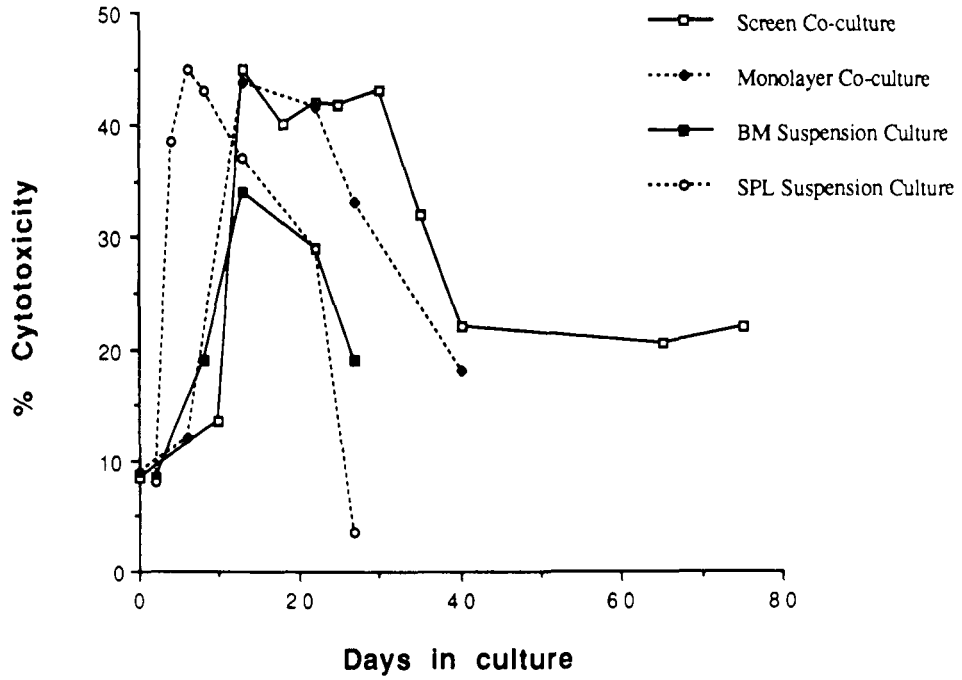

Figure 4. IL-2-induced cytolytic activity of cells generated from suspension cultures of nylon wool column nonadherent bone marrow cells or splenocytes, or suspended nylon screen or monolayer-based co-cultures against YAC-1 targets, as a function of time in culture. Co-cultures consisted of nylon wool column nonadherent bone marrow cells and spleen stromal cells.

Table IV. Comparison of Mean Percentage of Cytotoxicity ± 1 SE of Adherent and Quasiadherent Zone Cells of 12-Day Suspended Nylon Screen Cultures at Target to Effector Cell Ratios of 1:25 and 1:50

Target cells	Effector cells[a]			
	SPS & NK^{Ab}		SPS & NK^{QAc}	
	1:50	1:25	1:50	1:25
YAC-1	57 ± 2.8	24 ± 1.1	39 ± 1.5	20 ± 1.0
P-815	4.2 ± 2.1	0.5 ± 0.2	3.5 ± 1.9	1 ± 0.7

[a] Effectors were derived from enzyme-dissociated adherent or quasiadherent zone cells after passage through a packed nylon wool column. SPS, spleen stroma.
[b] Adherent NK (NK^{A}) cells derived from the adherent zone of suspended nylon screen cultures were collected, passed through packed nylon wool columns, and grown on monolayers of splenic stroma for 7 days in the presence of rIL-2. These cells represented 47 ± 2% of the total adherent cell count.
[c] Quasiadherent NK (NK^{QA}) cells were collected from the region between the nylon screen and the bottom of the tissue culture flask and grown on monolayers of splenic stroma for 7 days with rIL-2. Cytolytic activity in this zone represents those quasiadherent cells that were removed just prior to enzyme treatment of the nylon screen cultures to liberate adherent cells. The mean ratio of quasiadherent zone to adherent cell numbers was 1.23:1.

Table V. Mean Percentage of Cytotoxicity[a] ± 1 SE Generated by Cells from Suspended Nylon Screen Cultures[b] Established with Either Bone Marrow or Spleen Stroma and Activated after Incubation of Either Intact Screen Cultures or Enzyme-Dissociated Cells with rIL-2

Target cells	Effector cells[c]				
	SPS + NK		BMS + NK		NWP-BM[d]
	A[e]	B[f]	A[e]	B[f]	
YAC-1	49 ± 2.1	52 ± 3.1	46 ± 2.2	46 ± 2.4	40 ± 1.6
P-815	3.4 ± 2.1	4.0 ± 2.0	4.0 ± 1.9	3.0 ± 2.2	3.7 ± 1.5

[a] Assessed by ^{51}Cr release from target cells.
[b] Cells passed through packed nylon wool columns were cultured on splenic stromal cell monolayers in six-well plates for 6 days prior to being incubated with target cells.
[c] Effector cells were derived after passage through a packed nylon wool column. SPS, spleen stroma; BMS, bone marrow stroma.
[d] Freshly prepared nylon-wool-passed (NWP) bone marrow cells.
[e] A indicates intact nylon screen cultures treated with rIL-2, dissociated with enzyme, passed through a packed nylon wool column, and plated on SPS monolayers.
[f] B indicates that cells from nylon screen cultures were removed enzymatically, passed through a packed nylon wool column, plated on SPS monolayers, and treated with rIL-2.

range of maturation. NK 323, which is directed against a triggering structure involved with NK cell activation (30), appears to be expressed on immature as well as mature NK cells, since substantial labeling with this antibody was seen in the adherent zones, which continuously self-renewed the cultures for >4 months. In addition, the adherent zones contained an almost equal distribution of NK 323^{+} cells into IL-2R^{+} and IL-2R^{-} populations (Table II), even though the cultures were fed with IL-1, which induces IL-2R synthesis (37). Although the NK 323^{+}/IL-2R^{-} or −OX-8^{-} populations

probably represent immature NK cells, information concerning the phenotypic ontogeny of these cells in rodents is sparse. CD2 expression on the adherent zone cells varied from 15.3% to 25.8%. The expression of this epitope as related to NK cell maturation is uncertain as well; CD2 has been reported to contribute to certain mechanisms of NK cell activation (38, 39) and may, in some instances, be expressed on less mature NK cells.

IL-1 accentuates NK progenitor proliferation on stroma derived from either spleen or bone marrow (Fig. 3), but both entities were required for long-term NK cell culture. After IL-2 activation, IL-1-treated cultures of enriched bone marrow NK cells on spleen stroma contained >50% mature NK cells after >75 days in culture (Table II) and cytolytic activity was maintained over the same period (Fig. 4). Even though the seeding efficiency of nylon-wool-passed bone marrow cells on the suspended screen/stromal templates was only 45%, these cultures were more profilic than similar co-cultures established on stromal monolayers, where seeding efficiency was ~100% (Fig. 3) (36). However, both of these mixed culture systems were superior to suspension cultures, which lacked a pre-established stromal matrix. IL-1 induces the production of multilineage growth factors and colony-stimulating factors by stromal cells (40, 41) and initiates the proliferation of T lymphocytes under certain conditions (42). IL-1 also can stimulate the production of other cytokines such as IL-6, which markedly influences the growth and differentiation of T and B lymphocytes during immune responses (43, 44). Stimulation of T cell proliferation by accessory cell-derived IL-6 involves not only a direct growth-promoting signal, but also the induction of IL-2 receptors, an event that converts T cells to an IL-2-responsive state. IL-1 and IL-6 are strongly synergistic in the induction of the T cell proliferation (45). IL-1 enhanced both bone marrow and spleen stromal cell support of NK cell proliferation. A separate study indicates that IL-1 stimulates stromal and other cells to secrete IL-6 and multilineage growth factors (46). IL-6 also may directly influence NK cells by inducing IL-2 receptor synthesis (45), an event that permits the transformation of these cells into cytolytic killers by IL-2.

Murine monoclonal antibodies that recognize NK progenitor cells do not react with NK precursors or mature NK cells expressing IL-2 receptors (47). The more mature NK cells are concentrated in the spleen (35, 47). Our results are consistent with these findings. Thus, bone-marrow-derived nylon-wool-passed cells must be cultured for more than 10 days in order to generate cytolytic activity, whereas spleen-derived nylon-wool-passed cells require only 5–7 days of culture to become cytolytic, a finding that probably reflects the higher input level of the more mature spleen-derived NK cells. In contrast, there is a greater longevity in the proliferation of bone-marrow-derived cells as compared with NK cells isolated from the spleen. However, neither suspension culture system promotes the self-renewal of immature NK elements. In contrast, self-renewal of these cells in suspended nylon screen cultures can be inferred by the sustained production of NK cells for periods of >2 months. It appears that, in this system, NK precursors have a tendency to migrate into the quasiadherent zone as they differentiate. This would explain the ability of a large percentage of these quasiadherent cells to become actively cytolytic after exposure to IL-2. Since the mean ratio of NK cell numbers collected from the quasiadherent zone over time to the NK cells recovered from the adherent zone was 6.67:1, most of the cytolytic activity can be ascribed to the quasiadherent area.

Whereas the expression of IL-2 receptors may be an index of the maturity of the NK cell population, it may not correlate with the cytolytic activity displayed by these cells. IL-2 receptors have been associated with proliferation signal transduction by NK cells, but they do not appear to influence effector to target cell recognition (48, 49). In addition, discrepancies between NK cell phenotypic characterization and cytolytic function have been reported (50). The percentage of phenotypically identifiable NK cells in nylon screen cultures tended to increase with time, and approximately 50% of these cells expressed IL-2 receptors (Table II). However, cytolytic action against YAC-1 targets, which was maximal at 12–32 days in culture, declined by 40–45% to a level that was maintained from Day 40 to Day 75 of culture. This loss of cytolytic potential may be attributed to: (i) the less efficient separation of stromal cells in the longer-term cultures and subsequent dilution of NK cell numbers by these stroma during the incubation with target cells; (ii) the possibility that cultured NK cells may display a sensitivity to enzymatic digestion, which, although not altering phenotypic expression, may modify the ability of these cells to recognize targets; and (iii) the loss of the ability of some of the cultured NK cells to synthesize certain membrane proteins which may be necessary for the recognition of target cells.

Rat bone-marrow-derived nylon wool nonadherent cells proliferate and retain their cytolytic potential for extended periods if co-cultured with bone marrow or splenic stromal cells stimulated by IL-1. Previous studies have shown that bone marrow contains stroma that is capable of supporting murine B lymphopoiesis *in vitro* (21). The culture conditions, however, must be different than in Dexter long-term murine bone marrow cultures, which favor myelopoiesis (14, 17). In addition, stromal cells that support lymphopoiesis persist in long-term bone marrow cultures, and can re-establish this function if culture conditions are "switched" (51). In this regard, the most important

modifications to the medium are the elimination of both the steroid-rich horse serum conditioning and the hydrocortisone supplement. In contrast, suspended nylon screen bone marrow cultures support the synthesis of myeloid as well as lymphoid cells (22, 24) in medium supplemented with hydrocortisone in concentrations of 10^{-7}–10^{-8} M, which, in our experience, will not influence the mitogenic response of lymphocytes to either pokeweed mitogen or phytohemagglutinin (unpublished observations). However, hydrocortisone was present only in trace amounts in the medium used to feed the NK cell cultures. Overproliferation of the stromal support cells was not problematic, since the co-cultures were fed with medium containing ≤2.75% FBS and the hematopoietic cells were transferred to "fresh" nylon screen/stromal cell templates when the cultures started to become confluent. LGL cells displayed multidimensional growth patterns in the 210-μm sieve spaces and could be transferred to new nylon screen/stromal cell templates after enzyme dissociation and nylon wool column separation. The finding that the numbers of these cells steadily increased over input values following each successive re-inoculation is indirect evidence for the continued renewal of progenitors of this lineage. The relative percentages of cells that can be transformed into active cytolytic cells by IL-2 is similar, regardless of whether the nylon wool non-adherent cells are grown on stromal cell nylon screen templates or stromal monolayers. However, proliferation of the NK cells is substantially higher in the three-dimensional system; therefore, it produces greater absolute numbers of cytolytic cells than the monolayer-based co-cultures.

We are currently repeating these studies using human cells; if substantial numbers of NK/LAK cells can be generated *in vitro*, it may be possible to induce tumor regression in more patients given this therapy and to diminish the toxicity associated with LAK cell therapy by reducing the need for IL-2 *in vivo*. The possibility that hematopoietic organ stroma or stromal cells derived from tumor tissue might support the growth of tumor infiltrating lymphocytes is being investigated also. This is the first report indicating that a pre-established matrix of normal stromal cells can support NK cell proliferation *in vitro* and that the numbers of these cells can be expanded substantially for extended periods in culture with retention of cytolytic activity.

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