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# Feeder cells enhance oncolytic and proliferative activity of long-term human bone marrow interleukin-2 cultures and induce different lymphocyte subsets

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**Summary.** The effect of feeder cells on oncolytic activity of lymphocyte subsets and their growth was evaluated in long-term human bone marrow interleukin-2 (IL-2) cultures. Two B-lymphoblastoid cell lines (Daudi and Epstein-Barr-virus-transformed BSM) and two human leukemias, AML-M5, were used as feeder cells. The most prominent effects were seen in cultures stimulated with Daudi cells. In these cultures, cytotoxic activity was 100–1000 times increased against a broad range of target cells and the total cellular expansion was more than 40 times higher than in control cultures. This Daudi-related effect appeared to be mediated by natural killer (NK) cells, since cellular expansion occurred mostly in the $CD16^+$ and $CD56^+$ $CD3^-$ NK cell subset. In cultures stimulated with BSM and acute myelogenous leukemia (AML) feeder cells, the increase in proliferation was similar, but the enhancement of cytotoxicity, even though significant, was less prominent. Although all feeder cells were effective in stimulation of bone marrow reactivity, the highest cytotoxicity was always observed with feeder cells autologous to the targets, indicating some degree of specificity. This was especially evident in cultures stimulated with autologous versus allogeneic AML feeder cells. In contrast to Daudi-stimulated IL-2 cultures, in which the highest expansion of $CD3^-$ $CD56^+$ NK cells was observed, in BSM and AML cultures, the $CD3^+$ $CD56^{+/-}$ T cell subsets were more prolific. This indicates that the response and phenotypic heterogeneity of bone marrow cultures depends on the type of feeder cells used. This observation indicates that the preferential stimulation of a pertinent lymphocyte subset for therapeutic purposes may be possible.

**Key words:** Feeder cells – Bone marrow cultures – IL-2 – Lymphocyte subsets

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## Introduction

Natural killer (NK) cells have been recognized as the major contributing population to the lymphokine-activated killer (LAK) phenomenon [8, 10, 11, 14, 19]. The majority of human peripheral blood NK cells can proliferate and acquire high levels of cytotoxicity against a broad range of human tumor cells after activation with interleukin-2 (IL-2) [6, 8, 10, 11, 14, 19]. We and others have shown that purified peripheral blood IL-2-activated NK cells, in contrast to unseparated lymphocytes or T cells, will maintain high levels of proliferation and cytotoxicity over several weeks in culture [6, 19]. NK cells are characterized by the expression of the CD16 antigen (low-affinity receptor for the Fc portion of IgG), which they share with polymorphonuclear leukocytes [12, 17]. Almost all NK cells will also express the CD56 antigen but not the T cell-associated antigen, CD3 [6–8, 10, 11, 14, 19]. However, after activation with IL-2, two phenotypically distinct populations of NK cells can be found, both sharing the CD56 antigen but one lacking the CD16 antigen. The ratio of the two populations varies greatly among the individual donors [3].

Because of their high antitumor activity after IL-2 activation NK cells may be the major effectors within IL-2-activated peripheral blood lymphocytes (PBL) used for adoptive immunotherapy; however, on average the PBL of healthy donors will consist of only 15% NK cells, with large individual variability [12]. Several techniques have been described to enrich and/or preferentially expand NK cells in IL-2 cultures [5, 6, 13, 16]. A promising method for preferential NK cell expansion from peripheral blood has been reported recently [13]. This method consists of coculture of PBL with IL-2 and irradiated B lymphoblastoid cell lines, notably Daudi. Both IL-2 and irradiated feeder cells are required to obtain a high percentage of $CD16^+$ NK cells. In extension of these studies, a potent soluble factor, different from known lymphokines and enhancing cytotoxic activity, has been isolated from these cultures [4]. However, this factor does not seem to be responsible for the observed proliferative activity.

Although human PBL have been used successfully to generate cytotoxic effector cells for adoptive cellular immunotherapy, data from our laboratory and others suggest that it is possible to generate cytotoxic NK cells with equal or higher activity from bone marrow [1, 9]. In addition, bone marrow cells have been shown to purge contaminating tumor cells [1] successfully. Therefore, bone marrow cells may represent an interesting new avenue in adoptive cancer immunotherapy and in patients with leukemia or disseminated solid tumors receiving bone marrow transplants as a rescue after lethal doses of chemotherapy or radiotherapy. However, bone marrow samples available for this purpose usually contain only small numbers of cells for activation, which in addition contain a low percentage of NK cells. The present study was designed to test the ability of different feeder cells (allogeneic or autologous to the target cells) to enhance NK cell proliferation and cytotoxic activity of human bone marrow.

## Materials and methods

*Target cell and feeder cell lines.* The human Burkitt-lymphoma-derived cell line Daudi, the Epstein-Barr-virus-transformed B-lymphoblastoid cell line BSM and fresh human acute myelogenous leukemia blasts (AML-M5, obtained from peripheral blood of AML patients containing >80% blasts) were used as target and feeder cells. Additionally, the human ovarian carcinoma Ovcar-3 (NK-resistant) and the human erythromyeloid K-562 (NK-sensitive) cell lines were used as targets.

*Preparation of bone marrow cells.* Heparinized bone marrow from two normal donors and one breast carcinoma patient in remission was separated on Ficoll/Hypaque gradients as described previously [10]. After washing, the cells were adjusted at $10^6$/ml in culture medium. The cellular composition of the three bone marrow samples used (mean ± SE) was determined morphologically and was the following: 8.6±3.2% lymphocytes, 1.5±0.3% large granular lymphocytes, 0.4±0.05% blasts, 1.2±0.4% promyelocytes, 13.6±0.9% myelocytes, 23.0±1.5% metamyelocytes, 26.1±3.8% segmented neutrophils, 0.7±0.2% plasma cells, 0.9±0.4% eosinophils, 2.1±1.0% monocytes, 1.0±0.2% pronormoblasts and 21.8±2.8% normoblasts. The cancer patient bone marrow showed no diagnostic abnormalities and was free of tumor cells.

*Culture medium and growth conditions.* Bone marrow cells were cultured at $10^6$/ml in RPMI-1640 (Flow Laboratories, McLean, Va.) supplemented with 10% heat-inactivated human AB serum (Pel Freez Biological, Rogers, Arkansas), 10 mM HEPES buffer (Flow Laboratories, McLean, Va.), 2 mM glutamine (Gibco, Grand Island, N.Y.), 50 µg/ml streptomycin, 500 U/ml penicillin and 50 µg/ml gentamicin and $10^3$ U/ml recombinant human IL-2 (generous gift of Cetus Corp., Emeryville, Calif.) highly purified from *E. coli* with a specific activity of $18 \times 10^6$ IU/mg. Cultures were replaced twice weekly with fresh medium and the cell concentration readjusted to $10^6$/ml. To some cultures, irradiated (5000 R $^{60}$Co) allogeneic or autologous feeder cells were added together with IL-2 at the initiation of cultures (see Results). The autologous and allogeneic designation refers to the relationship between feeder and target-cells. The ratio of effector cells to feeder cells was 5 : 1.

*Target cell preparation.* K-562, Daudi, Ovcar-3 and BSM target-cells were maintained as continuous cultures in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum (Hazelton, Lenexa, Kan.), HEPES buffer, antibiotics and glutamine (s-RPMI). For use in the cytotoxicity assay, $2.5 \times 10^6$ K-562, Daudi, Ovcar-3 and BSM cells were labelled with 50 µCi $^{51}$Cr for 30 min at 37° C in 5% $CO_2$ humidified atmosphere. Frozen AML targets were thawed and incubated for 16 h in s-RPMI at 37° C and 5% $CO_2$; $5 \times 10^6$ viable AML were then labelled for 2 h with 200 µCi $^{51}$Cr. After labelling, all target-cells were washed with s-RPMI and adjusted to $10^5$ cells/ml. The viability of all targets was >80% (trypan blue exclusion test). Samples of $5 \times 10^3$ target cells (T) were incubated with effector cells (E) at 1 : 1, 1 : 3, 1 : 6, 1 : 12 and 1 : 25 T : E ratios.

*Cytotoxicity assay.* Cytotoxicity was tested in a 3-h $^{51}$Cr-release assay as described in detail previously [10]. Spontaneous $^{51}$Cr release was assessed by incubating target cells alone and ranged from 6% to 9% for cultured target cells and 6% to 18% for AML. The cytotoxicity was expressed in lytic untis (LU), 1 $LU_{30}$ being defined as the number of effector cells causing death of 30% of targets. The cytotoxicity was expressed per $10^7$ bone marrow cells and was calculated with an iterative non-linear-regression program provided by the BMDP software package [2] from the cytotoxicity data using five different E : T ratios (see above) and the Van Krogh model [15].

*Phenotype analysis.* Fluorescein-isothiocyanate- and phycoerythrin-labelled mouse anti-(human Ig) monoclonal antibodies (mAb) Leu11c (anti-CD16), Leu4 (anti-CD3) and Leu19 (anti-CD56; Becton-Dickinson, Mountain View, Calif.) were used. Mouse IGg1 and IgG2a served as controls. Samples of $5 \times 10^5$ cells were labelled for 30 min with 10 µl mAb, and the percentage of positive cells was determined with a FACSscan analyzer (Becton-Dickinson) within 24 h. Double staining of cells was achieved by simultaneous incubation with two mAb.

*Analysis of cell growth.* The total number of cells per culture was measured weekly; the cell viability (>90%) was analyzed by the trypan blue exclusion test. The cell growth was also monitored with a [$^3$H]dT assay; $10^6$ cells were plated in a 96-well microtiter plate (6 wells) and 50 µl Hanks' balanced salt solution containing, 0.5 µCi [$^3$H]dT (2 Ci/mmol specific activity, Amersham) was added to each well. The cells were incubated for 16 h with [$^3$H]dT at 37° C and 5% $CO_2$ and incorporated radioactivity was measured in a liquid scintillation counter (Beckman). The data were expressed as the mean ± SE of the 6 wells.

*Physical separation of bone marrow and feeder cells.* These experiments were performed with the same growth media as described above using a micropore well with a semipermeable bottom as a second compartment (0.2 µm, Millipore, Bedford, Mass.). Effector cells were plated in the wells of a 6-well plate (Falcon, Lincoln Park, N.J.) and feeder cells were plated in the micropore well, which was then immersed in the well containing effector cells; thus, both cell populations were exposed to the same culture medium without physical contact.

## Results

### *Effect of interleukin-2 and feeder cells on generation of human bone marrow cytotoxicity*

Cytotoxic activity of normal bone marrow cells stimulated with IL-2 alone or together with irradiated feeder cells was measured weekly for 5 weeks against K-562, Daudi, BSM and fresh AML. As shown by the experiments in Fig. 1 (representative of ten performed) the cytotoxicity of bone marrow cultures was greatly enhanced in the presence of feeder cells at all time intervals. The highest enhancement of cytotoxicity (100- to 1000-fold) against K-562, Daudi, Ovcar-3 and allogeneic AML was observed after stimulation with Daudi. BSM feeder cells were also effective in significantly enhancing cytotoxicity against these targets, but this enhancement was less prominent. Interestingly, Daudi, BSM and AML feeder cells also stimulated cytotoxicity against fresh AML.

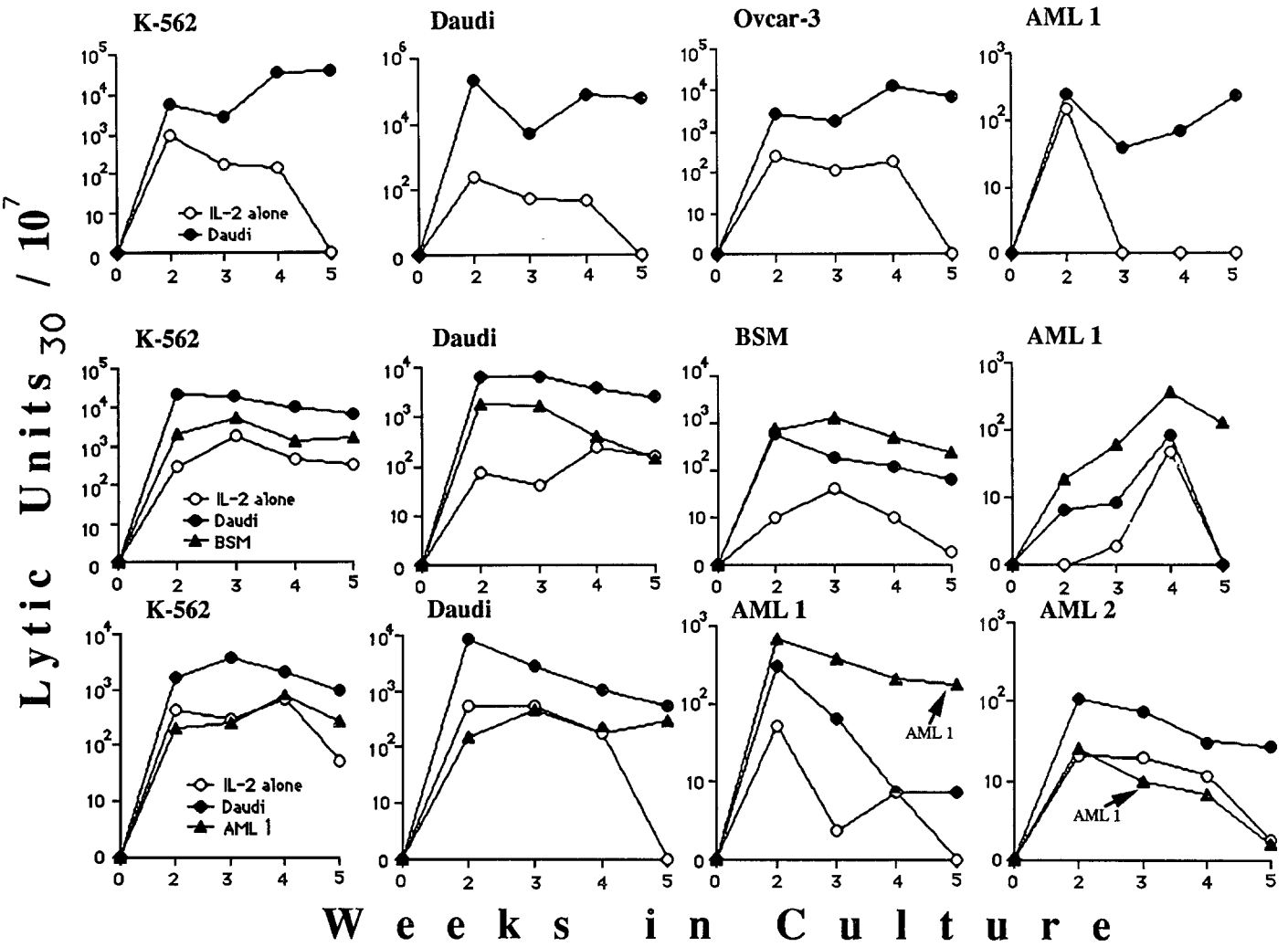


**Fig. 1.** Cytotoxic activity of human bone marrow cells from normal donors in long-term cultures with IL-2 and feeder cells. Data of three representative normal donors are shown

Even though allogeneic feeder cells were effective in generating the high degree of cytotoxicity, feeder cells autologous to the target cells were always most effective. This suggested that, in addition to unspecific activity, some specific responses were generated in bone marrow cultures supplemented with feeder cells. The cytotoxicity in most cultures (stimulated with feeder cells or IL-2 alone) peaked at 2 weeks; however, high levels of lysis were maintained in most feeder cell supplemented cultures up to 5 weeks. In contrast, in most of the bone marrow cultures stimulated with IL-2 alone, the cytotoxicity declined more rapidly.

*Inhibition of stimulatory effect by physical separation of feeder and bone marrow cells*

To study the mechanism of feeder cell stimulation, we separated these cells from bone marrow effector cells with micropore filters (0.2 μm) and tested for cytotoxic activity. The results, shown in Table 1, clearly indicate that physical separation did abrogate feeder cell stimulation of cytotoxicity of bone marrow cultures. This indicates that the mechanism of feeder cell stimulation and induction of the particular lymphocyte subsets is based on cell-to-cell contact rather than on a soluble substance.

*Effect of feeder cells on proliferation of human bone marrow cultures*

As illustrated in Fig. 2, the IL-2 bone marrow cultures stimulated with feeder cells exhibited significantly higher growth than those with IL-2 alone. In two representative experiments, the highest cell growth was seen in Daudi-stimulated cultures (41-fold increase, experiment A and 16-fold increase, experiment B). Somewhat less, but still substantially higher growth, was observed in cultures stimulated with BSM and AML (14-fold in experiments A and B). These results were reflected by [$^3$H]dT uptake, which was also increased in feeder-cell stimulated lymphocytes. In addition to showing increased proliferation, feeder-cell-stimulated lymphocytes persisted for a longer time in culture ($7.5 \pm 1.4$ weeks) when compared to lymphocytes stimulated with IL-2 alone ($4.5 \pm 1.3$ weeks) (data not shown).

**Table 1.** Cytotoxicity of bone marrow cells cultured with IL-2 and feeder cells with and without physical separation[a]

| Cultures | Separation | Cytotoxicity ($LU_{30}/10^7$ cells) | | | | | |
|---|---|---|---|---|---|---|---|
| | | K-562 | | Daudi | | BSM | |
| | | Expt. 1 | Expt. 2 | Expt. 1 | Expt. 2 | Expt. 1 | Expt. 2 |
| IL-2 | No | 202 | 105 | 39 | 55 | 2 | 6 |
| | Yes | 184 | 113 | 48 | 62 | 4 | 4 |
| IL-2 + Daudi | No | 3366 | 11781 | 1872 | 19701 | 180 | 372 |
| | Yes | 195 | 980 | 69 | 2410 | 3 | 21 |
| IL-2 + BSM | No | 1046 | 1891 | 639 | 1009 | 1786 | 2197 |
| | Yes | 247 | 596 | 28 | 198 | 20 | 57 |

[a] Data represent two experiments of 3-weeks bone marrow cultures. The technique for the physical separation of bone marrow and feeder cells is described in Materials and methods

*Phenotype of long-term human bone marrow cultures stimulated with interleukin-2 and feeder cells*

The bone marrow cultures with or without feeder cells were examined for distribution of CD16+ and CD56+ CD3− NK cells and CD56+ CD3+ and CD56− CD3+ T cells (Fig. 3). The weekly analysis (for 5 weeks) showed a substantial increase of CD16+ and CD56+ CD3− NK cells and CD56+ CD3+ T cells and a decline in the CD56− T cell subset in bone marrow cultures stimulated with IL-2 and Daudi (two representative experiments out of ten performed). This was in contrast to bone marrow cultures stimulated with IL-2 alone, which showed a predominance of CD56+ CD3+ and CD56+ CD3− subsets. To a somewhat lesser extent, an increase in NK cells was also observed in BSM- and AML-stimulated bone-marrow cultures; however, in these cultures the CD56+ CD3+ and CD56− CD3+ T cell subsets also predominated.

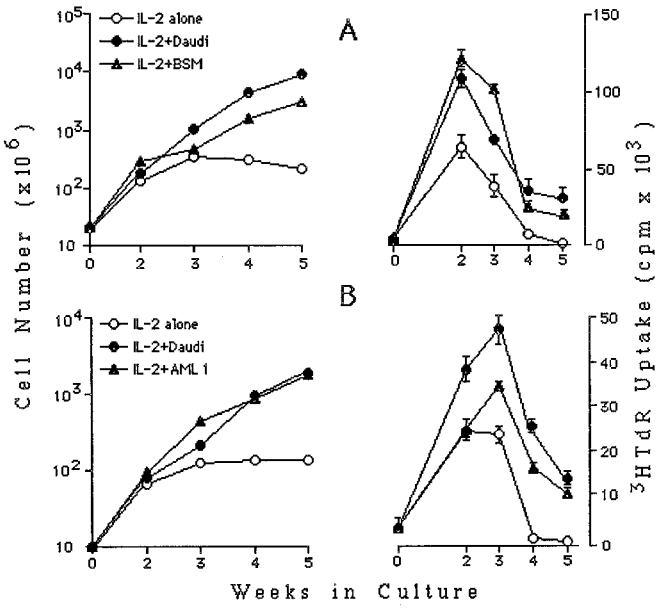


**Fig. 2.** Cell growth and [$^3$H]dT uptake of human bone marrow cultures stimulated with IL-2 and feeder cells. Two representative experiments are shown

*Analysis of the growth of NK and T cell subsets in human bone marrow cultures stimulated with IL-2 and feeder cells*

We analyzed the growth of NK (CD16+ and CD56+ CD3+) and T cell (CD3+ CD56− and CD3+ CD56+) subsets in bone marrow cultures stimulated with feeder cells and IL-2 at 5 weeks and compared the results to those from cultures stimulated with IL-2 only. As indicated in Table 2, stimulation of bone marrow cultures with Daudi cells resulted in the highest expansion of CD16+ (155-fold relative and 198-fold absolute increase) and CD56+ CD3− (126-fold augmentation index and 158-fold absolute increase) NK cells. Some expansion was observed also in the CD56+ CD3+ T cell subset (11-fold both the relative and absolute increase), whereas no major changes were seen in the CD3+ CD56− T cell subset. Increase in NK cell growth was also observed after stimulation with BSM and AML feeder cells; however, the effect was 5–10 times less than that observed in Daudi-stimulated cultures. Stimulation with the latter feeder cells also led to a substantial increase of CD3+ CD56+ and CD3+ CD56− T cells.

## Discussion

We have demonstrated that the proliferation and cytotoxic activity of IL-2-activated human bone marrow cells can be substantially enhanced by coculture with allogeneic and autologous (with respect to target cells) feeder cells. The highest proliferative activity was observed in cultures stimulated with Daudi cells and a slightly lower proliferative response with BSM and AML cells. It should be noted that the response was highly variable within the donor population, especially with AML cells.

The increase in cytotoxicity was highest in Daudi-stimulated bone marrow cultures. The range of tumor cells killed as well as the expansion of NK-cell subsets indicated that the enhanced cytotoxic activity is predominantly MHC-nonrestricted. However, the observation that the cytotoxicity in feeder-cell-stimulated cultures was always highest against autologous targets indicates that some of the observed cytotoxicity was specific. This observation is supported by the fact that, in addition to the expansion of

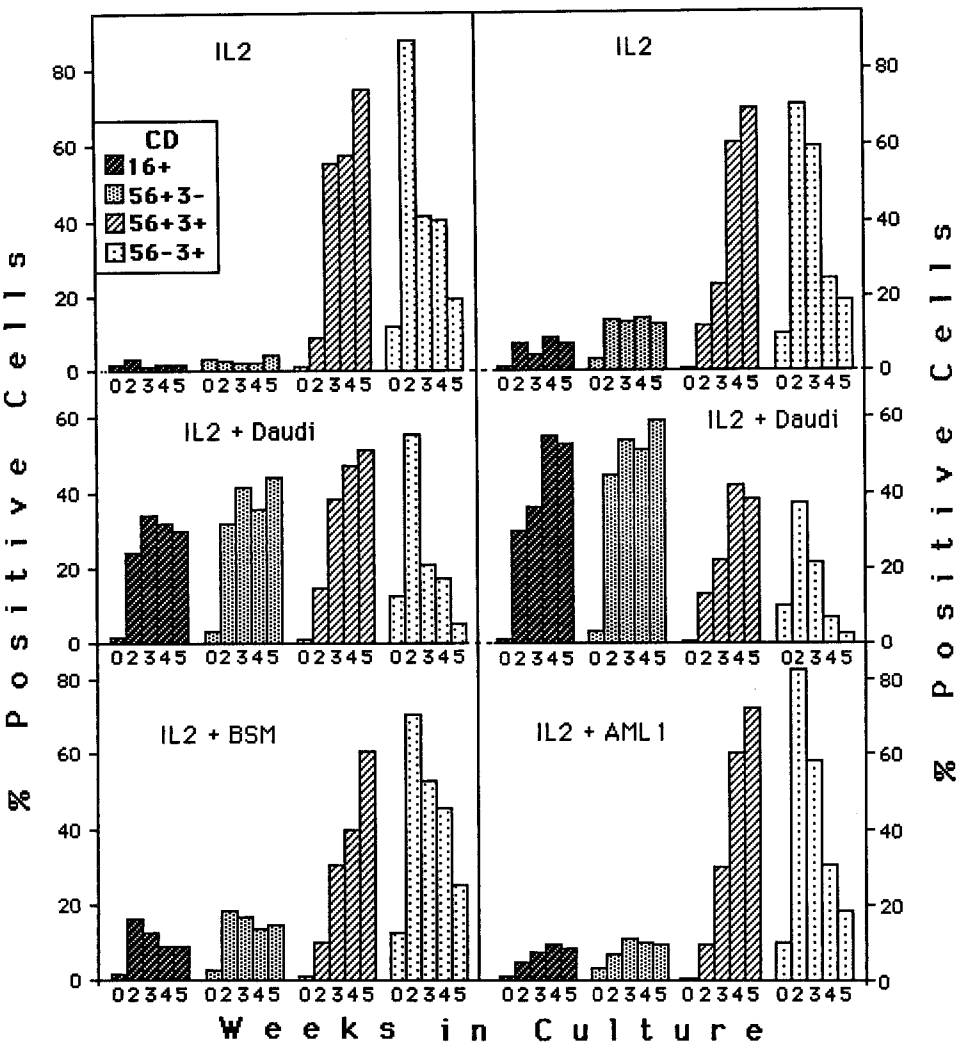


**Fig. 3.** Phenotype of long-term bone marrow cultures stimulated with IL-2 and allogeneic feeder cells. Two representative experiments are shown

NK cells, T cell subsets were also enhanced in feeder-cell-stimulated cultures. Although this is the first report using bone marrow cultures and multiple feeder cells, our results are similar to those with peripheral blood lymphocyte cultures, which were also found to be increased in both proliferative and cytotoxic activity after stimulation with Daudi feeder cells [13]. These studies are also comparable with those describing the generation of multiple lymphocyte subsets with different target specificities from peripheral blood of cancer patients [18].

We also found that the increased proliferative and functional response of IL-2-activated bone marrow cells to

**Table 2.** Growth of natural killer (NK) and T cell subsets in bone marrow cultures stimulated with interleukin-2 (IL-2) and feeder cells

| Parameter | NK cell subsets[c] | | T cell subsets[c] | |
|---|---|---|---|---|
| | $CD16^+$ | $CD56^+CD3^-$ | $CD3^+CD56^-$ | $CD3^+CD56^+$ |
| Augmentation index[a] | | | | |
| IL-2 | 38 ± 29 | 29 ± 21 | 26 ± 8 | 3234 ± 1619 |
| IL-2 + Daudi | 5888 ± 1191 | 3653 ± 1191 | 63 ± 33 | 36558 ± 13250 |
| IL-2 + BSM | 843 | 758 | 255 | 13254 |
| IL-2 + AML | 933 | 578 | 326 | 128961 |
| $10^{-6}$ × Absolute cell growth[b] | | | | |
| IL-2 | 6 ± 4 | 11 ± 5 | 66 ± 38 | 89 ± 45 |
| IL-2 + Daudi | 1188 ± 658 | 1743 ± 871 | 157 ± 62 | 995 ± 547 |
| IL-2 + BSM | 270 | 440 | 766 | 1856 |
| IL-2 + AML | 121 | 167 | 326 | 1289 |

[a] Augmentation index of 5-weeks bone marrow cultures was calculated as the mAb-positive cell number at maximum expansion of culture/mAb-positive cell number at start of culture (day 0)

[b] Absolute cell growth ($\times 10^{-6}$) for each subset calculated as total cell number at maximum expansion of culture × mAb-positive cells (%) at maximum expansion

[c] The data represents a mean ± SE of three experiments with the exception of BSM (1 experiment only)

feeder cells required the initial intimate contact between both types of cell, since their physical separation at the initiation of the cultures did not produce this effect. These observations again correspond to those seen with human PBL by others [13]. However, the exact mechanism of action of feeder-cell-stimulatory activity is not known. A previously unidentified potent factor has been isolated in PBL cultures activated with B-lymphoblastoid feeder cells and IL-2 [4], but this factor seems to be only responsible for the enhancement of cytotoxic activity.

Human activated bone marrow has been recently identified as a potent host versus tumor effector in transplantation [1]. Our report indicates, that it is possible to generate high numbers of highly activated MHC-nonrestricted NK cells and T cells with a broad range of antitumor activity from human bone marrow, by using allogeneic feeder cells, namely Daudi. In addition, large numbers of target-specific and highly cytotoxic bone marrow cells can be generated with autologous feeder cells, as shown here with fresh human AML cells. Such bone marrow cultures, particularly those stimulated with irradiated AML, may be useful in clinical immunotherapy with patients undergoing bone marrow transplantation and/or with hematological malignancies.

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