

# LYMPHOKINE-ACTIVATED KILLER CELLS

## Analysis of Progenitors and Effectors

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A number of previous reports (1–4) have indicated that recombinant and hybrid IFN, as well as natural IFN vary considerably in their ability to mediate biological activities. Recent studies (4, 5) have also shown that NK activity can be significantly augmented by natural and recombinant species of IFN- $\alpha$ . In addition to IFN, IL-2 has been reported (6–10) to play a major role in many types of immune responses. This lymphokine induces proliferation of T lymphocytes and NK cells and the production of IFN- $\gamma$ ; it also results in the induction of lymphokine-activated killer (LAK)<sup>1</sup> cells against previously NK-resistant cell preparations and cell lines (7–11). LAK cells induced by IL-2 are potent cytotoxic cells that may have an important role in immunotherapy of solid tumors.

In the studies reported here, we separated normal lymphocytes into large granular lymphocytes (LGL) and T lymphocyte subsets by fractionation on Percoll density gradients. Selected leukocyte subpopulations were subsequently depleted with antibody and immunoadsorbent techniques. After purifying leukocyte subsets, we determined which of them generated LAK activity in the presence of IL-2. Similarly, we were able to determine the phenotype of LAK effector cells by generating LAK activity from unseparated peripheral blood mononuclear cells and then selectively depleting various lymphocyte subsets. In addition, we used a sensitive limiting-dilution frequency assay to examine unseparated leukocytes and subpopulations of lymphoid cells for their ability to act as progenitors and effectors of LAK activity.

Finally, because of recent reports (11) regarding the role of the T cell receptor in mediating cytolytic activity against target cells, we examined the effect of antibodies against a CD3 molecule as well as other molecules to study their possible relationship to the generation of cytotoxic activity.

### Materials and Methods

*Preparation of Target Cells.* The following tumor cell lines were used as targets in the cytotoxicity assay: K562, derived from a patient with chronic myelogenous leukemia in

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<sup>1</sup>*Abbreviations used in this paper:* IAP, immunoadsorbent plates; LAK, lymphokine-activated killer; LGL, large granular lymphocyte; LDA, limiting-dilution analysis; OVCA, ovarian carcinoma.

blast crisis (12); MBL2, a Moloney leukemia virus-transformed cell line (13); FEMX, a human melanoma cell line (14); and fresh, noncultured, human tumor cells obtained from frozen aliquots isolated from ascites fluid of a patient with ovarian adenocarcinoma. Aliquots of fresh tumor cells were frozen and used for individual assays (6). Target cells were cultured at 37°C in a humidified atmosphere of air with 5% CO<sub>2</sub> in RPMI 1640 medium (Biofluids, Inc., Rockville, MD) supplemented with 10% human AB serum (Gibco, Grand Island, NY), 0.3 mg/ml glutamine, 100 IU/ml penicillin, and 10 µg/ml streptomycin. The culture lines were initiated at 0.3 × 10<sup>6</sup> cells/ml. When cell density reached or approached 2 × 10<sup>6</sup> cells/ml, we centrifuged the cells at 120 g for 10 min, discarded the supernatant, and collected the cell pellet.

*Isolation of LGL and T Lymphocytes.* LGL and T lymphocytes were obtained from buffy coats of peripheral blood from normal healthy volunteers, as previously described (15, 16). Briefly, mononuclear cells were separated by centrifugation on a Ficoll-Hypaque gradient and depleted of monocytes by adherence on a plastic surface. Nonadherent cells were applied to a nylon wool column, and the eluted cells were then fractionated on a seven-step discontinuous gradient of Percoll (Pharmacia Fine Chemicals, Uppsala, Sweden) at concentrations of 40–60% (15). LGL were collected from the low-density fractions (fractions 2 and 3, counting from the top), whereas T cells were found in the higher-density bottom fraction. Contaminating T lymphocytes were further removed from the LGL fraction by rosetting with SRBC at 29°C for 1 h (16). The LGL, which do not form rosettes at this temperature, were recovered from the interface of a Ficoll-Hypaque gradient. LGL-enriched preparations contained an average of 80–90% LGL, as determined by morphological analysis of Giemsa-stained cytocentrifuge preparations, and were 70–75% OKM1<sup>+</sup>, 75–80% CDw16<sup>+</sup> (Leu-11), and <5% CD3<sup>+</sup> cells, as determined by flow cytometry (16). Hereafter, this cell preparation will be referred to as the unfractionated or whole LGL population. Purified T lymphocyte preparations contained <1% LGL and were >95% CD3<sup>+</sup> (16).

*Cytotoxicity Assay.* Target cells were labeled with 100 µCi <sup>51</sup>Cr (New England Nuclear, Boston, MA) for 1 h at 37°C, and extensively washed before use. Target (5 × 10<sup>3</sup>) and effector cells in 0.1 ml of medium were plated in microtiter plates at several E/T ratios (25:1, 12:1, 6:1, 3:1). After a 4-h incubation at 37°C, the supernatants were harvested and counted in a gamma counter. Cytotoxicity was determined by the amount of <sup>51</sup>Cr released from dead target cells. Three replicates were used for each experimental group, and the percent specific lysis was calculated as 100 × [(cpm in experimental wells) – (cpm in wells with target cells alone)] / (cpm incorporated into target cells). A lytic unit (LU) is defined as the number of cells mediating 30% lysis (6). An increase in isotope-release levels of 6% above baseline was consistently statistically significant (*p* < 0.05 Student's *t* test).

*Culture Medium.* Culture medium was RPMI 1640 supplemented with 10% human AB serum (M. A. Bioproducts, Walkersville, MD), 0.1 mM nonessential amino acids (Gibco), 2 mM sodium pyruvate (Gibco), 4 mM glutamine (National Institutes of Health Media Unit, Bethesda, MD), 50 µg/ml gentamicin (Sigma Chemical Co., St. Louis, MO), and 2.5 × 10<sup>-5</sup> M 2ME (Sigma Chemical Co.). This medium will be referred to hereafter as complete medium.

*Treatment and Separation of Cells with mAbs.* The following mAbs were used in this study: CD3 (OKT3, Leu-4) (pan-T antigen), CD8 (OKT8; suppressor/cytotoxic T cell), CD4 (OKT4; helper/inducer T cells), CDw16 (3G8; Fcγ receptor on LGL and neutrophils), and NKH1 (reactive on most LGL; a gift from Coulter Immunology, Hialeah, FL) and Leu-M3 (monocytes) was used as a control Ig reagent.

Cells were treated with mAbs according to the following procedures: 5 × 10<sup>6</sup> LGL were labeled with 20 µl mAb at the concentration of 1 µg/ml in 0.1 ml of PBS with 1% FCS (Gibco) for 45 min at 4°C. At the end of the incubation, the cells were washed twice with PBS.

*Separation of Lymphocyte Subsets with Immunoabsorbent Plates (IAP).* Lymphocyte subsets were separated on an anti-F(ab')<sub>2</sub> IAP by modification of the methods previously described (18, 19). Affinity-purified F(ab')<sub>2</sub> goat anti-mouse IgG (Cappel Laboratories, West Ches-

ter, PA or Boehringer Mannheim, Indianapolis, IN), at 10 mg/ml, was immobilized on 7-cm plastic dishes for 2–18 h at 4°C. Binding efficiency of the protein ranged from 40 to 70%, as determined by measuring the reduction in the optical density of the protein solution with a spectrophotometer (Gilford Instrument Laboratories, Inc., Oberlin, OH) at 280 nm (after the coupling). Before use, plates were washed with RPMI 1640 containing 10% FCS and incubated at 37°C for 30 min. mAb-treated cells, at  $10^7$  cells/ml (total of 5 ml/dish) were put onto the IAP. Plates were incubated for 60 min at 4°C. Medium plus EDTA (1 mM) was gently poured on top of the plate, and negative (nonadherent) cells were collected in 10–15 ml. Positive (adherent) cells were detached using a cell scraper, and collected by washing. Reanalysis of the isolated population revealed that positive fractions contained <2% inappropriate cells, whereas negative fractions contained up to 10%.

**Limiting-dilution Analysis (LDA).** In the LDA, culture medium was supplemented with 500 U/ml rIL-2 obtained from Biogen, Inc. (Cambridge, MA). LDA were performed as previously described (20). Briefly, responder cells (ranging from 10,000 to 10 cells/well, 12 replicates per concentration) were dispensed in 0.1 ml of complete medium containing IL-2 into round-bottom, 96-well plates (Linbro Chemical Co., Hamden, CT). Autologous irradiated (3,000 rad) mononuclear cells ( $2 \times 10^5$  cells/well) were added in 0.1 ml. Each well received a total volume of 0.2 ml. Plates were cultured for 7 d to determine the frequency of proliferating cells, and for 8 d in studies of cytotoxic precursor frequency. These incubation times have been found to be optimal for such studies (17). To determine frequency of cytotoxic progenitor cells, plates were washed in medium without IL-2 6–8 h before the cytotoxicity assay to reduce possible nonspecific lysis (9, 10). Medium (0.1 ml) was replaced with  $^{51}\text{Cr}$ -labeled target cells ( $5 \times 10^3$  cells/well).

Proliferation was assessed by measuring the cells' uptake of [ $^3\text{H}$ ]thymidine (sp act, 6.7 mCi/mmol; New England Nuclear) during the last 18 h of incubation. Each well received 1  $\mu\text{Ci}$  of [ $^3\text{H}$ ]thymidine. The cells were then harvested onto glass fiber discs with an automatic harvester, and radioactivity was measured in a liquid scintillation counter.

**Assessment of LDA Results.** Background control values were determined by measuring the uptake of [ $^3\text{H}$ ]thymidine and release of  $^{51}\text{Cr}$  in wells (24 replicates) containing autologous feeder cells with medium plus IL-2. Test wells were considered positive when values exceeded the mean control value by three standard deviations. Using linear regression analysis, the minimum estimate of frequencies was calculated as the number of responder cells plated relative to the log of the percentage of negative wells (37%). The significance of differences between different lines of responder cells within the same experiment was calculated as described by Taswell (20).

## Results

**Characterization of LAK Precursors.** Leukocyte subpopulations were fractionated and tested for their ability to generate LAK activity after 3–5 d of incubation in rIL-2. Initially, enriched populations of LGL and T cells were prepared from mononuclear PBL (Table I). In more than 10 experiments, the LGL-rich fractions consistently demonstrated higher levels of rIL-2-generated LAK activity against fresh ovarian carcinoma (OVCA) cells and FEMX (human melanoma cells), than did the T cell-enriched fractions. We also saw high levels of activity against NK-sensitive K562 cells. To further characterize the precursor cells capable of generating LAK cells, subsets of PBL enriched to express CDw16 or CD3 were cultured in rIL-2 for 3–4 d (Table II). LAK activity was generated from several of these subsets. As with the Percoll-isolated cells, the LGL-rich populations (CDw16<sup>+</sup>, NKH1<sup>+</sup>) were the most active. However, the T cell-rich population, CD3<sup>+</sup>, also had considerable cytotoxic activity. Cytotoxic activity against NK-sensitive and -resistant targets could be efficiently generated in Fc $\gamma$ R<sup>-</sup> LGL after 3–5 d in culture with rIL-2. LAK cells generated from unseparated

TABLE I  
LAK Activity in Subpopulations of Leukocytes Isolated on Percoll Gradients

| Cell type | Cells staining positively on day 4 for:* |        |      | Cytolytic activity <sup>‡</sup> |      |      |
|-----------|--|--------|------|---------------------------------|------|------|
|           | CD3                                      | Leu-11 | NKH1 | K562                            | OVCA | FEMX |
|           | %  |        |      | LU/10 <sup>7</sup> cells        |      |      |
| PBL       | 90                                       | 6      | 8    | 401                             | 9.0  | 38   |
| LGL       | 4  | 63     | 83   | 725                             | 35   | 109  |
| T         | ≥97                                      | ≤1     | 3    | 301                             | 12   | 9    |

\* Surface markers were analyzed by flow cytometry.

‡ Cytotoxic activity in a 4-h <sup>51</sup>Cr-release assay.

TABLE II  
LAK Activity in Subpopulations of Leukocytes Isolated with mAbs

| Leukocyte sub-population* | Cells staining positively for markers on:‡ |       |      |       |       |      | Cytolytic activity against target cells:‡ |          |                          |          |                          |          |
|---------------------------|--|-------|------|-------|-------|------|---|----------|--------------------------|----------|--------------------------|----------|
|                           | Day 0                                      |       |      | Day 4 |       |      | K562                                      |          | FEMX                     |          | OVCA                     |          |
|                           | CD3  | CDw16 | NKH1 | CD3   | CDw16 | NKH1 | LU/10 <sup>7</sup> cells                  | Total LU | LU/10 <sup>7</sup> cells | Total LU | LU/10 <sup>7</sup> cells | Total LU |
|                           | %  |       |      |       |       |      |   |          |                          |          |                          |          |
| PBL                       | 95   | 8     | 10   | 96    | 12    | 14   | 207                                       | 175      | 59                       | 28       | 16                       | 14       |
| CD3 <sup>+</sup>          | ≥98  | ≤2    | ≤2   | ≥98   | ≤2    | 3    | 15  | 32       | 17                       | 43       | 12                       | 35       |
| CD3 <sup>-</sup>          | 11   | 94    | ≥97  | 8     | 59    | 95   | 307                                       | 209      | 108                      | 76       | 60                       | 43       |
| CDw16 <sup>+</sup>        | ≤2   | ≥97   | ≥97  | 7     | 59    | 84   | 285                                       | 192      | 125                      | 88       | 81                       | 50       |
| CDw16 <sup>-</sup>        | ≥97  | ≤2    | ≤2   | 92    | ≤2    | 4    | 22  | 40       | 10                       | 31       | 8                        | 21       |

\* Leukocyte subpopulation isolated by IAP.

‡ Percent positive cells determined by flow cytometry.

§ LU/10<sup>7</sup> effector cells, of phenotype indicated, measured in a 4-h <sup>51</sup>Cr-release assay. Total lytic units was calculated from cell recovery.

peripheral blood mononuclear cells or purified T cells also mediated significant cytotoxic activity, but required substantially more effector cells to mediate similar levels of lysis. When the ability of the various phenotypically characterized subsets to mediate cytotoxicity is analyzed on the basis of the total amount of cytotoxic activity, it is clear that the contribution of T cells to the total number of lytic units in the population is variable (depending on target cell), but is generally in the range of 15–50% of the total cytotoxic activity, and therefore the LGL fraction routinely contributes >50% of the total cytotoxic activity. Based on these results, physical separation of cells could result in isolation of the majority of LAK activity within a minor lymphocyte subset. Fig. 1 graphically demonstrates the relative contributions of LGL and T cells to the generation of LAK activity. If one examines cell recovery and lytic activity against FEMX cells, one can see that a 45.8% Percoll cut-off results in a restriction of 69% of the total lytic activity within 13.7% of the total number of cells. This finding was consistent in several experiments, where the majority (>60%) of the lytic activity was restricted to 15–20% of the total number of cells (generally, those cells that are isolated from 46% Percoll fractions).

Further studies were performed to exclude the possibility that activity generated from T cells was due to contamination by NK cells. CDw16<sup>+</sup> and OKM1<sup>+</sup> cells were depleted from the Percoll-isolated T cell population. Treating the T

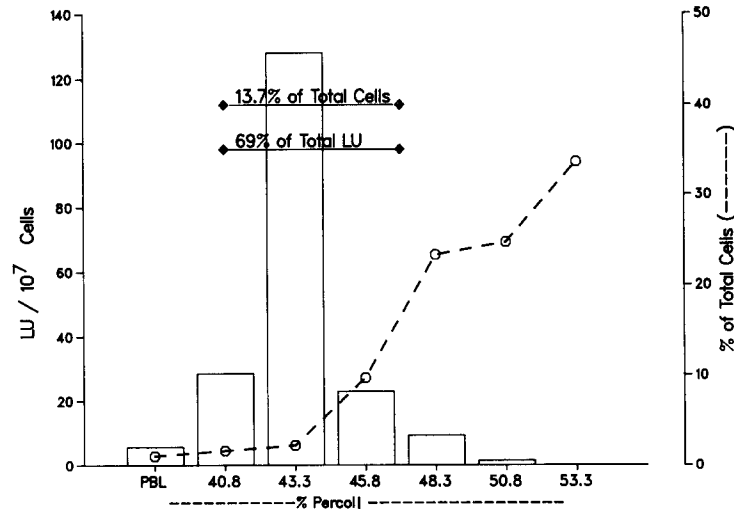


FIGURE 1. Relative activity level of Percoll-isolated lymphocytes. Figure shows LU/10<sup>7</sup> cells (bars) as well as the total cell recovery (○). The total LU and the total cell recovery are summarized. The results show cytotoxic activity against FEMX target cells after 4 d incubation with 500 U/ml human rIL-2.

cell fraction with antibodies routinely used to deplete NK cells did not alter the ability of CD3<sup>+</sup> T cells to generate LAK activity (data not shown).

**Kinetics of LAK Generation by LGL and T Cell Subpopulations.** The data presented above demonstrate that both T cells and LGL contributed to rIL-2-generated LAK activity. Therefore, we investigated whether the kinetics of LAK cell generation with rIL-2 differed between these subpopulations. LGL and T cells were cultured for 1–5 d in 500 U/ml IL-2-containing medium. Interestingly, the PBL and T cell populations were consistently slower to generate LAK activity, with the maximal level of activity appearing on day 2 or later (Fig. 2). However, when LGL-enriched populations were used, high levels of cytolytic activity were seen against tumor cells by day 1. These results indicate either that LGL can be activated more rapidly than T cells or that cell-cell interactions occur in the LGL population such that the development of LAK activity is optimized. Therefore, it appears that activated NK and T cells kill fresh tumor cells, but that these two cell types generate those activities at different rates.

**Frequency of LAK Progenitors.** To directly examine the actual progenitor frequencies of these various populations, limiting-dilution frequency analysis was performed. Fig. 3 illustrates a typical result, where unseparated lymphocyte populations and various subsets of cells isolated on Percoll density gradients were examined for their ability to generate LAK cells. These cells were plated in a limiting-dilution analysis on day 0, and their cytotoxic activity was analyzed on day 4. As in other experiments, the activity level was highest in the first three Percoll fractions. The results of these assays also directly demonstrate that the frequency of progenitors is highest in the first two fractions from Percoll density gradients. In comparison to the unseparated lymphocyte populations, there is a ~10–50-fold increase in the number of progenitors present in fractions 1 and 2. Fractions 4, 5, and 6, which are primarily T cell-enriched populations, generate

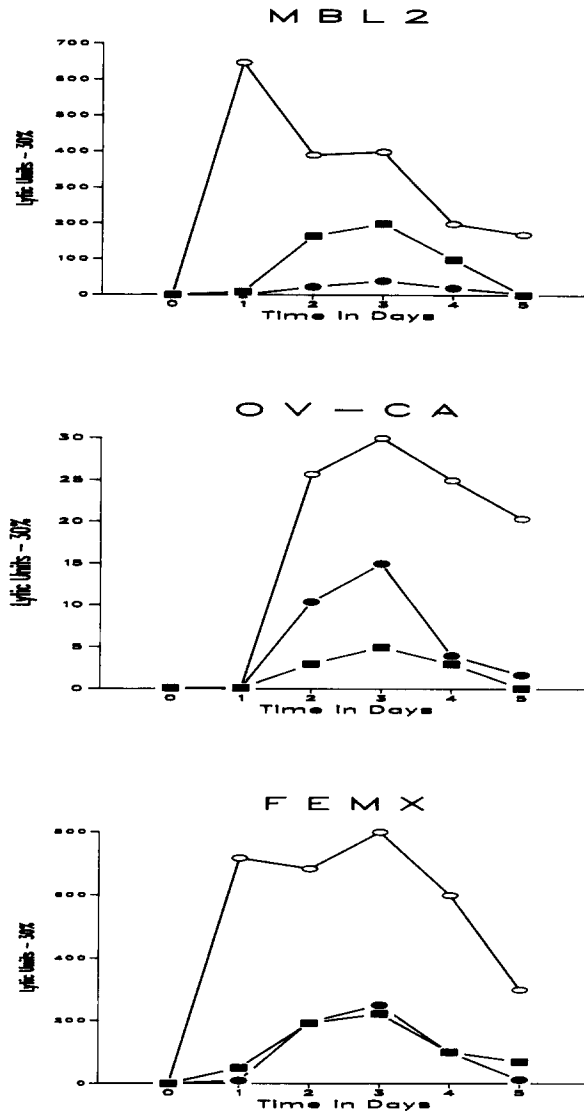


FIGURE 2. Kinetics of LAK generation by LGL and T cell subsets. The kinetics of lysis of OVCA, MBL2, and FEMX cells by unseparated lymphocyte (■) and Percoll-isolated LGL (○) or T cell (●) fractions are shown.

precursor frequencies ( $\sim 1/10^5$ ) similar to those observed in the unseparated populations. Therefore, the low-density (LGL-enriched) fractions are capable of generating more cytotoxic activity (as measured in LU) and the frequencies of progenitor cells are considerably higher in this population than in the unseparated populations or in other subsets of cells prepared from Percoll gradients.

*Characterization of Effector Cells Mediating LAK Activity.* Experiments were performed to analyze the phenotype(s) of cells capable of mediating the broad-spectrum cytotoxic activity generated when unseparated lymphoid cells were cultured in rIL-2. The data presented in Table III were obtained after peripheral

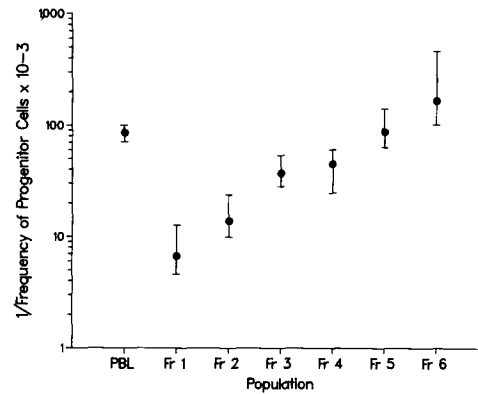


FIGURE 3. Frequency analysis of LAK progenitor. Limiting-dilution frequency analysis of LAK progenitor cells was performed on day 0. The effector function of the various dilutions was measured for cytotoxicity against FEMX target cells on day 4. Results show the frequency ( $\pm 95\%$  confidence interval) based on limiting-dilution  $\chi^2$  analysis.

TABLE III  
*Characterization of Effector Cells Mediating LAK Activity*

| Effector cells from: | Cells staining positively for: |     |      | Cytolytic activity against: |          |                          |          |
|----------------------|--------------------------------|-----|------|-----------------------------|----------|--------------------------|----------|
|                      | CD3                            | CD8 | NKH1 | MBL2                        |          | OVCA                     |          |
|                      |                                |     |      | LU/10 <sup>7</sup> cells    | Total LU | LU/10 <sup>7</sup> cells | Total LU |
|                      | %                              |     |      |                             |          |                          |          |
| Unseparated PBL      | 95                             | 34  | 7    | 25.8                        | 68       | 16                       | 28       |
| CD3 <sup>+</sup>     | 98                             | 36  | 8    | 1.7                         | 1.3      | 12                       | 35       |
| CD3 <sup>-</sup>     | 14                             | 11  | 97   | 108                         | 76       | 60                       | 43       |
| CD8 <sup>+</sup>     | 97                             | 87  | 85   | 1                           | <1       | <1                       | <1       |
| CD8 <sup>-</sup>     | 96                             | 12  | 24   | 16                          | 5        | 6                        | 2        |

Characterizations performed 4 d after IL-2 activation of effector cells separated by IAP on day 0.

blood mononuclear cells had been cultured for 4 d in the presence of 500 U/ml rIL-2. The cultured effector cells were divided into CD3<sup>+</sup> and CD3<sup>-</sup> or CD8<sup>+</sup> and CD8<sup>-</sup> sets. Analysis of the cytotoxic activity of these various subsets against NK-susceptible K562 cells and NK-resistant MBL2 and fresh human OVCA cells showed that CD3<sup>-</sup> lymphocytes mediated the highest levels of cytotoxicity against all three targets (data not shown). Interestingly, a high percentage (97%) of these CD3<sup>-</sup> cells bear the NKH1 marker. In contrast, the CD3<sup>+</sup> lymphocyte subset showed lower but consistent levels of cytotoxic activity. The CD8<sup>+</sup> lymphocyte subset appeared to contribute no cytotoxic activity against either of the NK-resistant target cells, however, considerable loss of activity was seen compared to unseparated PBL. To further investigate the role of CD8<sup>+</sup> effector cell, purified T cells were cultured for 4 d and then separated into CD4<sup>+</sup> or CD4<sup>-</sup> subsets. Table IV shows a representative experiment indicating that CD8<sup>+</sup> effector cells were active against OVCA, whereas CD8<sup>-</sup>, CD4<sup>+</sup> cells were devoid of significant cytolytic activity. However, when the total contribution to cytotoxic activity of

TABLE IV  
Characterization of T Effector Cells Mediating LAK Activity

| Effector cells from: | Cells staining positively for: |     |     | Cytolytic activity against OVCA |          |
|----------------------|--------------------------------|-----|-----|---------------------------------|----------|
|                      | CD3                            | CD4 | CD8 | LU/10 <sup>7</sup> cells        | Total LU |
|                      | %                              |     |     |                                 |          |
| Unseparated T        | 98                             | 45  | 52  | 6.3                             | 30       |
| CD4 <sup>+</sup>     | 97                             | ND  | 7   | <0.1                            | <1       |
| CD4 <sup>-</sup>     | 97                             | 3   | 89  | 30.6                            | 41       |

Characterizations performed 4 d after IL-2 activation. See previous tables for details.

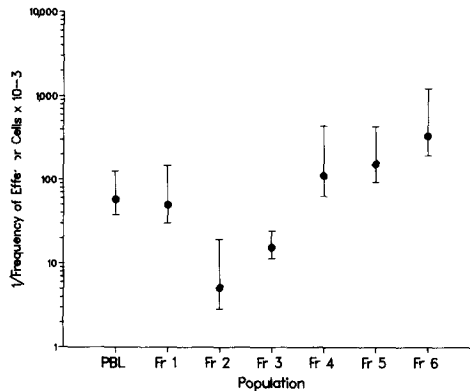


FIGURE 4. Frequency analysis of LAK effector. Limiting-dilution frequency analysis of LAK effector cells was performed on day 4. The effector function of the various dilutions was measured for cytotoxicity against FEMX target cells on day 4. Results show the frequency ( $\pm$  95% confidence interval) based on limiting-dilution  $\chi^2$  analysis.

the various subsets was calculated, it was apparent that the CD3<sup>+</sup> and CD3<sup>-</sup> population contributed significantly to the lysis of fresh ovarian carcinoma cells.

**Effector Cell Frequency Analysis.** To directly examine the phenotype and frequency of effector cells in various populations of cells, unseparated peripheral blood mononuclear cells were cultured after depletion of monocytes or B cells, or after fractionation on discontinuous Percoll gradients. Cells were cultured for 4 d in bulk cultures at  $4 \times 10^6$  cells/ml, and the effector frequency was determined using limiting-dilution frequency analysis at day 4. A typical result of this type of experiment is shown in Fig. 4. As in the direct cytotoxicity assays, considerably more effector cells were present in the low-density LGL-enriched fractions than in the high-density fractions, which are predominantly composed of CD3<sup>+</sup> T cells. Again, based on a number of experiments, it is clear that, generally, 10–40 times more effector cells are found in the low-density Percoll fractions than in the high-density, T cell-enriched fractionated lymphocytes, indicating a high level of effector frequency in LGL (CDw16<sup>+</sup>, NKH1<sup>+</sup>) effector populations.

**Analysis of Effector Cell Receptors Involved in Mediating Lysis.** Because two phenotypically distinct lymphocyte subsets mediate lytic activity against NK-susceptible target cells, we wished to determine which effector cell receptors



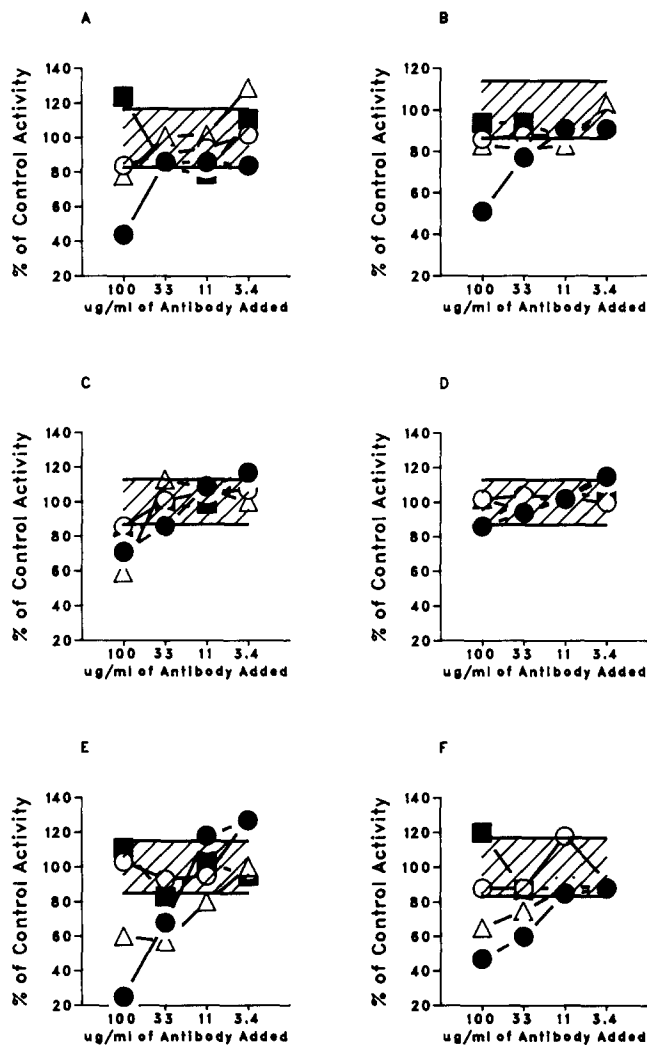


FIGURE 5. Effect of anti-CD determinants on LAK activity in PBL, LGL, and T cells. PBL (A and B), LGL (C and D), and T cells (E and F) were cultured for 4 d, and their NK (A, C, and E; tested against K562) and LAK (B, D, and F; tested against FEMX) activity was measured after various amounts of anti-CD3 (●), anti-CD4 (○), anti-CD8 (△), and Leu-M3 (■) were added. The hatched area represents the percentage of control activity ( $\pm 1.5$  SD;  $p \leq 0.05$ ). Activity was measured in a 4-h  $^{51}\text{Cr}$ -release assay with the antibodies present during the entire assay.

might be involved. Therefore, antibodies directed against the CD3, CD4, CD8, CDw16, and NKH1 markers, some of which have been shown (11) to be involved in and to inhibit various cytotoxic activities of fresh T cells and T cell clones, were assayed against NK-susceptible K562 target cells and FEMX cells. A representative experiment is shown in Fig. 5. Antibody to Leu-M3, a monocyte-specific IgG antibody, was used as a control to measure nonspecific inhibition by antibody. If we used either FEMX or K562 target cells, we saw significant specific inhibition of cytotoxicity with CD3 determinant reagent, especially in populations

containing a high percentage of T cells. In addition, CD8 determinant antibodies moderately inhibited LAK (FEMX) and NK (K562) target cells, especially in T cell-enriched populations. However, LGL cytotoxicity was not inhibited to any significant extent, against either K562 or FEMX cells. Antibodies directed to CD16 or NKH1 had no effect against any target (data not shown). Although some inhibition of LGL-mediated lysis was seen with the CD3 and CD8 determinants, this level was not significantly different than that demonstrated by the Leu-M3 (IgG) control, and was only apparent at the highest dose administered (100  $\mu$ g/ml). Therefore, it appears that most of the activity mediated by T cells is regulated through the T cell receptor complex, whereas the LGL fraction is not inhibited by antibodies directed against the CD3 or CD8 determinants.

### Discussion

By definition, cytolysis mediated by LAK cells does not occur without stimulation. In contrast, NK cells purified on Percoll density gradients have some lytic activity against selected cultured and fresh tumor cells, but this has been demonstrated (11) against only a limited number of tumors. However, the target cell specificity of LAK cells and activated NK cells is very similar; both cell types can kill fresh tumor cell lines, 1,3,5-trinitrophenyl-modified cells, and virus-infected cells. LAK cells are stimulated mainly by IL-2, whereas NK cells are regulated by IL-1, and various species of IFN, as well as IL-2 (6, 11).

The study described here involved an in-depth analysis of the cells that generate LAK activity. This analysis of progenitor cells relied on phenotypic characterization, separation on Percoll gradients, and LDA to determine progenitor frequency. We have demonstrated that CD3<sup>+</sup> and CD3<sup>-</sup> cells can generate cytotoxic cells, termed LAK cells, from normal PBL. Although the effector cell that is isolated from whole PBL varies somewhat (depending on the target cell), the primary effector cell is coincident with the LGL population on day 0, the CDw16<sup>+</sup>, NKH1<sup>+</sup>, activated NK cell.

The nature of the bone marrow progenitor phenotype of NK cells has been extensively studied, and differs considerably from the mature NK cell phenotype. The major NK progenitor cell has been characterized as having a phenotype of CD3<sup>-</sup>, CD8<sup>-</sup>, OKT11<sup>-</sup>, OKM1<sup>-</sup>, CDw16<sup>-</sup>, and NKH1<sup>-</sup> (17). Our studies with limiting-dilution frequency analysis, which accurately assesses the frequency of a progenitor, are in partial agreement with the earlier report of Grimm et al. (8). However, our studies have indicated that more than one effector cell population is involved in generating what is known as LAK activity. Clearly, a CD3<sup>+</sup> cell contributes a small but significant amount to rIL-2-generated cytotoxicity. However, an LGL population that is CD3<sup>-</sup>, CD8<sup>-</sup> but bears the CDw16 and NKH1 markers contributes most of the rIL-2-generated LAK activity. This latter finding is consistent with a recent report of cytolytic activity of CDw16<sup>+</sup>-activated killers (10) against fresh melanoma tissue (10). The effector phenotype of LAK cells generated from unseparated PBL was originally reported to be CD3<sup>+</sup> and T11<sup>+</sup> (8).

Our results on effector cell frequency obtained using positive and negative selection procedures, and LDA, indicate that the actual cytotoxic effector cells are of a considerably different phenotype than was previously reported (7-9).

The effector frequency that is measured after lymphokine activation is highest in the low-density CD3<sup>-</sup> populations. Lytic unit calculations of cytotoxicity against fresh tumor cells and NK-resistant tumor cell lines such as FEMX yield the same results. The reason for the discrepancy between this and previous reports (7–9) is not clear; however, it may relate to the techniques that were used to analyze effector cells. These investigators (7–9) have used complement-dependent lysis to deplete populations of the desired effector cell. The ability of complement to lyse cultured cells, and the problems associated with modulating surface markers *in vitro* may have accounted for some of the discrepancy between our results and those of other researchers (10). However, by using direct isolation procedures, and by reanalyzing the populations used as effector cells, our study has clearly indicated that this LAK effect does not involve a single effector cell. In contrast to previous reports, the major effector cell does not appear to bear the CD3 marker associated with the T cell receptor, but seems to be a CD3<sup>-</sup> activated killer cell that closely resembles the NK cell, in that CDw16 and NKH1 markers are present.

The kinetics of cytotoxicity generation differ somewhat in these populations. CD3<sup>+</sup> cells require 2–3 d to generate maximal activity, whereas LGL-enriched populations are efficiently activated and show high levels of killing against both cultured and fresh tumor cells within 24 h of IL-2 treatment.

It is unlikely that a single cell type serves as the LAK progenitor, because both CD3<sup>+</sup>, CDw16<sup>-</sup> and CD3<sup>-</sup>, CDw16<sup>+</sup> cells can be activated by rIL-2. These subsets also mediate LAK activity to different degrees. Therefore, the overall results suggest that LAK is not generated from a unique precursor or mediated by a unique effector. As a result, we conclude that LAK cells are generated from several different lymphocyte precursors, and that the cytotoxic effector cell population consists of several different cell types that contribute to the overall cytotoxicity, with LGL contributing the major portion of the activity.

Our analysis of the receptors involved in the activation of these various subset populations supports the conclusion that several cell types generate LAK. This is especially obvious when the CD3-directed mAb are used to inhibit cytotoxic activity. These CD3 antibodies have been shown (11) to inhibit cultured as well as cloned populations of cytotoxic T lymphocytes that interact with the CD3 determinant, which is a part of the T cell receptor. Therefore, these results allow us to conclude that T cell receptor-associated cytotoxicity is responsible for part of what is known as LAK activity, but that most LAK activity, on a per-cell basis, occurs through a cytolytic function presumably mediated by T cell receptor-negative LGL (NK cells). Although our data do not identify an NK receptor, they do indicate a difference from T cells and the lack of T cell receptor involvement. Although an NK cell receptor has not been defined to date, the activity of these effectors and the parallel with T cell killing via the T cell receptor supports the contention that an NK cell receptor exists.

In summary, we have defined and characterized the lymphocyte populations responsible for generating and mediating the cytotoxicity known as LAK activity, and have shown that different receptors on the different cell types are involved in expression of LAK activity.

### Summary

IL-2 has been examined for its ability to regulate lymphokine-activated killer (LAK) activity. IL-2 is a potent activator of cytolytic activity against a wide array of tumor cells, including those from fresh autologous and allogeneic tumors. Using subpopulations of lymphoid cells that were separated on Percoll density gradients, and subsequently purified by immunoadsorbance, studies were performed to examine the phenotypes of progenitor and effector cells of human LAK cells and to compare them with the phenotype of activated NK cells. From these studies, it was evident that several lymphoid subsets, including CD3<sup>+</sup>, CDw16<sup>-</sup> and CD3<sup>-</sup>, CDw16<sup>+</sup> cells could mediate LAK lysis of fresh tumor cells. Our examination of the kinetics of activation revealed that CDw16<sup>+</sup>, NKH1<sup>+</sup> (NK-active) cells were maximally activated by 1–2 d. In contrast, CD3<sup>+</sup> cells appeared not to achieve maximal cytolytic activity against fresh and cultured tumor cells until days 2–3. Using limiting-dilution frequency analysis, we showed that a large percentage of cytolytically active progenitors was present among the CDw16<sup>+</sup>, NKH1<sup>+</sup> cells. The progenitor and effector cell frequencies appear to be 10–50 times higher in these populations compared to CD3<sup>+</sup> cells. In addition, the selective blockage by mAb to the CD3 determinant of the T cell receptor complex indicated that these two effector cell phenotypes relied on different receptors to mediate their cytotoxic activity against tumor cells. Therefore, the accumulated data suggest that there is not a single unique progenitor of LAK activity, but rather that multiple subsets of lymphocytes become cytotoxic in response to IL-2. However, the NK cell population forms the largest single component of LAK cell activity in human peripheral blood.

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