Activated killer cells can be generated by the incubation of peripheral blood mononuclear leukocytes (PBL) in the lymphokine interleukin 2 (IL-2). Unseparated populations of these lymphokine-activated killer (LAK) cells lyse a variety of fresh noncultured human tumor targets, but they do not kill normal PBL. This study analyzed the generation and lytic specificity of LAK cell clones. Of 49 (84%) clones isolated by limiting-dilution techniques from a whole population of LAK cells, 41 manifested significant LAK cell activity. LAK cell clones had varied cell surface phenotypes. Clones with high and intermediate LAK cell activity were Leu 2 3 4 + DR + Tac + and Leu 2 3 4 + DR + Tac +, respectively. Single LAK cell clones lysed multiple fresh human tumor targets including autologous sarcoma, 5 allogeneic sarcomas, and a colon cancer in addition to the cultured cell line K562. Autologous PBL were not lysed. Tumor targets were each lysed by multiple LAK cell clones. Sixteen subclones were derived from 5 of these LAK cell clones. These subclones had 99% or greater probability of being derived from a single cell. These subclones also exhibited lysis of multiple tumor targets. These findings suggest the existence of a shared determinant, expressed by multiple human tumors, which is recognized in common by multiple LAK cell clones.—JNCI 1985; 76:67-75.

ABSTRACT—Activated killer cells are generated by the incubation of peripheral blood mononuclear leukocytes (PBL) in the lymphokine interleukin 2 (IL-2). Unseparated populations of these lymphokine-activated killer (LAK) cells lyse a variety of fresh noncultured human tumor targets, but they do not kill normal PBL. This study analyzed the generation and lytic specificity of LAK cell clones. Of 49 (84%) clones isolated by limiting-dilution techniques from a whole population of LAK cells, 41 manifested significant LAK cell activity. LAK cell clones had varied cell surface phenotypes. Clones with high and intermediate LAK cell activity were Leu 2 3 4 + DR + Tac + and Leu 2 3 4 + DR + Tac +, respectively. Single LAK cell clones lysed multiple fresh human tumor targets including autologous sarcoma, 5 allogeneic sarcomas, and a colon cancer in addition to the cultured cell line K562. Autologous PBL were not lysed. Tumor targets were each lysed by multiple LAK cell clones. Sixteen subclones were derived from 5 of these LAK cell clones. These subclones had 99% or greater probability of being derived from a single cell. These subclones also exhibited lysis of multiple tumor targets. These findings suggest the existence of a shared determinant, expressed by multiple human tumors, which is recognized in common by multiple LAK cell clones.—JNCI 1985; 76:67-75.

Activated killer cells can be generated by the incubation of PBL in the lymphokine IL-2 (1, 2). These populations of LAK cells lyse both autologous and allogeneic NK cell-resistant tumor target cells in a standard 4-hour 51Cr release assay, while sparing normal tissue (2, 3). A broad range of fresh, noncultured human tumors of varied histologies is susceptible to lysis by LAK cells (4). We have demonstrated that LAK cells constitute a cytolytic T-cell system distinct from the classical CTL and NK cell systems (2, 5). Many investigators have now reported on the cytotoxicity mediated by human lymphocytes activated by incubation in IL-2-containing media (6-9). Other investigators have reported the existence of non-CTL cytolytic cell systems activated by allosensitization (10-19), autologous mixed lymphocyte reaction (20), mixed lymphocyte tumor culture (12, 21), interferon (22, 23), lectins (14, 24, 25), bacterial extracts (26, 27), and xenogeneic serum (28). Since most of these systems involve either culture in IL-2-containing media or activation processes that are known, or are likely, to induce IL-2 production, LAK cells may be the common effector cells for these systems.

The observation that LAK cell populations kill multiple tumor targets raises two alternative explanations for this polyspecificity: a) Individual LAK cells recognize and kill multiple tumor targets, or b) subpopulations of LAK cells exist that specifically lyse individual tumors.

In this report we have attempted to distinguish between these possibilities by cloning a population of LAK cells and analyzing cytotoxicity at the clonal level. We have also studied the cell surface phenotype of LAK cell clones and the frequency of LAK cells among the clonogenic cells of PBL incubated in IL-2.

MATERIALS AND METHODS

Media.—CM consisted of RPMI-1640 medium (GIBCO, Grand Island, NY) containing 10% heat-inactivated human AB serum (KC Biologicals, Kansas City, MO), 300 μg glutamine/ml (NIH Media Unit), 100 μg penicillin/ml (NIH Media Unit), 100 μg streptomycin/ml (NIH Media Unit), 50 μg gentamicin sulfate/ml (M.A. Bioproducts, Walkersville, MD), and 0.25 μg amphotericin/ml (Fungizone; Squibb, Flow Laboratories, McLean, VA).

Cryopreservation.—Cryopreservation of all cells was in 90% human AB serum plus 10% dimethyl sulfoxide. The vials containing 107 cells in 1 ml were placed in 95% ethanol at room temperature and transferred to a Revco freezer at -70°C. After a minimum of 4 hours the vials were then transferred to a liquid nitrogen freezer for long-term storage.

ABBREVIATIONS USED: CM=complete medium (media); CS=cloning suspension; CTL=cytotoxic thymus-derived lymphocyte(s); HBS=Hanks' balanced salt solution; IF=immunofluorescence; IL-2=interleukin 2; PP-IL-2=partially purified IL-2; LAK=lymphokine-activated killer; NIH=National Institutes of Health; NK=natural killer; PBL=peripheral blood mononuclear leukocyte(s); PHA=phytohemagglutinin; SB=staining buffer.

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5 We are grateful to Mr. Donald White for assistance with statistical analysis and to Ms. Susan Sharrow and Mr. David Stephany for flow cytometric analysis.
PBL.—PBL were obtained by fractionation of peripheral blood on lymphocyte separation medium gradients (Litton Bionetics, Inc., Kensington, MD) as previously described (450 X g for 20 min) (1). The cells were washed twice with HBSS and resuspended in CM. PBL were cryopreserved and subsequently thawed when needed.

IL-2.—PP-IL-2 is a lectin-free supernatant of PHA-stimulated lymphocytes (T-cell growth factor, IL-2, delectinatated; Cellular Products Inc., Buffalo, NY).

Activation of LAK cells.—PBL were activated to generate LAK cells by in vitro incubation for 5 days in CM containing PP-IL-2 (1:10, vol/vol). The PBL were activated in 25-cm² flasks (#3050; Costar, Cambridge, MA) in 10 ml of activation medium at a concentration of 10⁶ cells/ml. The flasks were incubated upright for 5 days in 5% CO₂ at 37°C.

In vitro growth of LAK cells.—Growth medium consisted of CM with the addition of PP-IL-2 (1:10, vol/vol) and 0.1 µg PHA/ml (Burroughs-Wellcome Ltd., Beckenham, England). Cells were cultured in 24-well plates (#3524; Costar). Feeder cells consisting of irradiated (3,000 rad) pooled PBL from 4 normal donors were added at a concentration of 4X10⁶ cells/2-ml well. Plates were incubated in 5% CO₂ at 37°C. We expanded the cells by transferring aliquots to wells with fresh medium, IL-2, PHA, and feeder cells. Cells were washed twice in CM prior to use as effectors in cytotoxicity assays.

Fresh tumor targets.—Single-cell suspensions of solid tumors were prepared by a modified version of our previously described technique (24, 29). Resected tumor was collected from surgery and transported in HBSS. Necrotic tumor and connective tissue were removed, and the remaining specimen was minced with scissors. The fragments were dissociated by mechanical stirring overnight in a flask containing CM (without AB serum) with 2 mg collagenase/ml (type IV; Sigma Chemical Co., St. Louis, MO), 5 U hyaluronidase/ml (type V; Sigma Chemical Co.), and 0.2-0.3 mg deoxyribonuclease/ml (type I; Sigma Chemical Co.). The dissociated cells were passed through steel mesh and washed in HBSS, first at 750Xg for 5 minutes and then at 175Xg for 5 minutes. Debris, nonviable cells, and nontumor cells were separated from viable tumor cells by centrifugation with the use of a standard lymphocyte separation medium gradient (450Xg for 20 min). The tumor cells were collected from the interface, washed twice, and resuspended in CM. An aliquot was sent for cytopathologic analysis, and the rest was cryopreserved. Samples were analyzed by the NIH Laboratory of Cytopathology, the Clinical Center, with the use of Papanicolaou staining to determine the percentage of tumor cells. The fresh tumor targets utilized in cytotoxicity assays in this paper included 2 malignant fibrous histiocytomas (sarcomas #1 and 2), a spindle cell sarcoma (sarcoma #3), an osteogenic sarcoma (sarcoma #4), an alveolar rhabdomyosarcoma (sarcoma #5), a synovial cell sarcoma (sarcoma #6), a malignant schwannoma (sarcoma #7), a colon adenocarcinoma, and a T-cell lymphoma.

Cultured tumor target.—K562, the NK cell-sensitive erythroleukemia cell line, was cultured in CM and used as a target either directly from culture or after cryopreservation.

Measurement of cytotoxicity.—A 4-hour ⁵¹Cr release assay was used to measure LAK cell killing of target cells. Target cells were thawed the morning of the assay and labeled with 400 µCi of Na₂⁵¹CrO₄ (Amersham Corporation, Arlington Heights, IL) for 120 minutes in 0.5 ml of CM at 37°C. The cells were washed twice in CM and incubated for an additional 0.5-1.0 hour at 37°C prior to a final wash. Target cells were passed through nylon mesh to eliminate aggregates and were added at 5X10⁵ cells in 0.1 ml CM to wells containing 0.1 ml CM and various numbers of effector cells in round-bottom microtiter plates (Linbro Chemical Co., Hamden, CT). The plates were centrifuged at 45Xg for 5 minutes and then incubated for 4 hours at 37°C in 5% CO₂. After incubation the plates were centrifuged at 175Xg for 5 minutes. The supernatants were harvested and counted in a gamma counter. Maximum isotope release was produced by incubation of the targets with 0.1 N HCl. Spontaneous release was determined by incubation of targets with CM alone. The percentage of specific lysis was calculated by the following formula: [(experimental cpm - spontaneous cpm)/(maximal cpm - spontaneous cpm)]X100%. All determinations were made in triplicate, and data are reported as the mean ± SEM.

Cloning.—Cloning was performed with the use of limiting-dilution techniques modified from our previously published methods (30). CS consisted of CM with the addition of PP-IL-2 (1:10, vol/vol), PHA (0.1 µg/ml), and irradiated feeder PBL (4 pooled normal allogeneic donors, 3,000 rad, 5X10⁶ cells/ml). The cells to be cloned were appropriately diluted in CS and delivered in 0.2-ml aliquots to individual wells of 2 (flat-bottom 96-well microtiter plates (#3596; Costar) for each cell per well dilution. Plates were incubated at 37°C in 5% CO₂. Fresh CS (50 µl) without feeder cells was added to each well on day 4. Culture supernatant (100 µl) was aspirated from each well on day 8 and replaced with fresh CS (including feeder cells). Plates were visually scored on day 12 or 13. Wells with growth were plucked and expanded as detailed in “Materials and Methods” for in vitro growth.

Monoclonal antibody staining and flow cytometry analysis.—The IF profile of cells was determined by the binding of fluorescein isothiocyanate-conjugated monoclonal antibodies. The staining reagents included Leu 2 (class I reactive T-cells), Leu 3 (class II reactive T-cells), Leu 4 (pan T-cells), Leu 7 (NK cells), Leu DR (la-bearing cells), and as a negative control Thy 1.1 (murine T-cells). These reagents were purchased from Becton Dickinson Monoclonal Antibody Center (Mountain View, CA). Anti-Tac monoclonal antibody (directed against the IL-2 receptor) was provided by Dr. Thomas Waldmann (National Cancer Institute) and was directly fluoresceinated following purification from ascites. To block nonspecific staining, we initially suspended cells in 100% human AB serum at a concentration of 2-10X10⁶ cells/ml. Then 1 ml of this suspension was aliquoted

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into 12X75-mm plastic tubes (#2052; Falcon Plastics, Oxnard, CA) and centrifuged at 110Xg for 5 minutes at 4°C. SB consisted of HBSS (without phenol red), 0.1% NaN3, and 0.02% bovine serum albumin (Miles Laboratory, Inc., Elkhart, IN). The supernatant was completely removed from tubes following each centrifugation. Added to the tubes was 15 μl staining reagent, diluted in SB at 4°C. Tubes were vigorously mixed and incubated in the dark for 45 minutes at 4°C. Following 2 washes in SB, cells were resuspended in 0.4 ml of SB and kept on ice until analyzed. Flow cytometry was performed as previously described with the use of an FACS II (Becton-Dickinson RACS Systems; Becton, Dickinson & Co., Sunnyvale, CA) (37). Fluorescence data for 20,000-50,000 viable cells were analyzed with the use of a PDP 11/34 computer (Digital Equipment Corp., Maynard, MA). IF profiles are presented as cell frequency histograms. The X-axis is the log (fluorescence intensity), and the Y-axis is the cell number.

RESULTS

Cytotoxicity of the LAK Cell Population

The PBL from a patient with a malignant fibrous histiocytoma (sarcoma #1) were incubated for 5 days in CM containing PP-IL-2 to generate LAK cells as described in "Materials and Methods." Viable cell recovery after activation was 66% of the initial number of PBL. The unseparated population of LAK cells was tested for cytotoxicity with the use of a 4-hour 51Cr release assay against a panel of targets including fresh autologous tumor and PBL, 5 allogeneic sarcomas, a colon cancer, a lymphoma, and the cultured NK cell-sensitive target K562 (table 1). Fresh PBL that were from the sarcoma #1 patient and that had not been activated by IL-2 were tested in parallel. The LAK cell population produced 67% lysis of the autologous sarcoma, 33-66% lysis of the allogeneic tumors, and 84% lysis of K562. The fresh PBL produced 25% lysis of K562, confirming the presence of NK cell activity, but they were not capable of killing fresh, noncultured tumor. The PBL target was not lysed by the LAK cells, but it was lysed by a CTL population that had been sensitized in vitro to that PBL target. This assay confirmed our previous findings that populations of LAK cells manifest polyspecific lysis of multiple NK cell-resistant fresh tumor targets, sparing normal PBL targets.

Growth of the LAK Cell Population

Cultures of this unseparated population of LAK cells were initiated at a concentration of 10^5 cells/ml in the presence of PP-IL-2, PHA, and irradiated feeder cells. These cultures expanded continuously for over 2.5 months prior to senescence. Meticulous attention to these cultures was required to obtain sustained growth. Cell viability in the cultures decreased within 12-18 hours after cell density exceeded 10^6 cells/ml. The cultures required splitting at a median of 4-day intervals; fresh feeder cells were added at each culture split. The total number of population doublings was 57. The LAK cell population maintained cytotoxicity throughout the period of expansion, as demonstrated by serial assays against the autologous tumor target (table 2).

Cloning of the LAK Cell Population

The unseparated population of LAK cells was cloned, with the use of the limiting-dilution techniques, at the completion of the 5-day activation in PP-IL-2. Thirteen days after cloning the plates were visually scored to determine the number of wells with growth. Regression analysis demonstrated a linear correlation of log(% negative wells) with the number of cells plated per well (P=.004). With the use of the Poisson analysis, wells with growth plated at 1 cell per well had an 89% probability of derivation from a single cell. Wells with growth plated at 3 cells per well had an 83% probability of clonality.

<table>
<thead>
<tr>
<th>Table 1.—Polyspecific killing of fresh human tumor targets mediated by unseparated populations of LAK cells</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Effectors</strong></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>LAK cells^&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>PBL^&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>CTL^&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Media, cpm</td>
</tr>
<tr>
<td>HCl cpm</td>
</tr>
</tbody>
</table>

^<sup>a</sup>Results are expressed as % specific lysis ± SEM. The effector-to-target cell ratio was 40:1. Significant lysis P<.05: LAK—all targets except PBL; PBL—only K562; CTL—sarcomas #1-4; PBL—colon cancer.

^<sup>b</sup>The unseparated populations of LAK cells from the patient with sarcoma #1 were used as effectors in a standard 4-hr 51Cr release assay against fresh (NK cell-resistant) tumor targets, PBL, and the cultured NK target K562.

^<sup>c</sup>Fresh PBL from the patient with sarcoma #1.
TABLE 2.—Maintenance of LAK cell cytotoxicity during culture in IL-2

<table>
<thead>
<tr>
<th>Effectors</th>
<th>Effector-to-target cell ratio</th>
<th>Days in culture of autologous sarcoma target*</th>
</tr>
</thead>
<tbody>
<tr>
<td>LAK cells</td>
<td>40:1</td>
<td>72±5</td>
</tr>
<tr>
<td></td>
<td>10:1</td>
<td>72±2c</td>
</tr>
<tr>
<td></td>
<td></td>
<td>77±3</td>
</tr>
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<td></td>
<td></td>
<td>67±3</td>
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<td></td>
<td></td>
<td>70±4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>63±4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>52±10</td>
</tr>
<tr>
<td>Media, cpm</td>
<td></td>
<td>31±5</td>
</tr>
<tr>
<td>HCl, cpm</td>
<td></td>
<td>42±9</td>
</tr>
<tr>
<td></td>
<td></td>
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<td>37±1</td>
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<tr>
<td></td>
<td></td>
<td>51±7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>35±5</td>
</tr>
</tbody>
</table>

* Results are expressed as % specific lysis of autologous tumor ± SEM. All lysis was significant (P<.05).

The unseparated LAK cell population was used as effector cells in a series of 4-hr 51Cr release assays against the autologous sarcoma target. 20:1, effector-to-target cell ratio.

5:1, effector-to-target cell ratio.

Manifestation of LAK Cell Activity by Clones

Text-figure 1 presents the results of the initial testing of all clones for LAK activity. Of the 39 wells with growth on the 1-cell-per-well plates, 26 could be successfully expanded. At initial testing, 73% (19/26) of these clones demonstrated LAK cell activity (>10% specific lysis of autologous tumor). From the 3-cells-per-well plates, 91% (21/23) of expanded clones had LAK cell activity. Despite interassay variation on retesting 4–13 days later, most clones retained comparable levels of LAK cytotoxicity, though several clones without initial cytotoxicity developed substantial lysis when retested. Autologous PBL were not killed by the clones (0/22 tests). These assays demonstrated that most clones derived from the unseparated LAK cell population have LAK cell activity and that there is a range of LAK cell activity for the clones. Clones can be identified with high or intermediate to low LAK cell activity.

Growth of LAK Cell Clones

Ten clones from the 1-cell-per-well plates were expanded for further analysis. The kinetics of growth for the clones were similar to those for the unseparated population. Text-figure 2 shows the growth curves for 2 of the clones, one with intermediate LAK activity (1AE12) and the other with high LAK activity (1BH10). The total in vitro life-span of the clones extended to 76 days, but it
LAK Cell Clones

Lysis by LAK cell clones of multiple fresh human tumor targets

<table>
<thead>
<tr>
<th>Effectors</th>
<th>Autologous</th>
<th>Targets</th>
<th>Allogeneic</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sarcoma #1</td>
<td>Sarcoma #2</td>
<td>Sarcoma #3</td>
</tr>
<tr>
<td>1BA6</td>
<td>55±1</td>
<td>41±4</td>
<td>68±5</td>
</tr>
<tr>
<td>1BH10</td>
<td>55±3</td>
<td>38±2</td>
<td>62±3</td>
</tr>
<tr>
<td>1AA2</td>
<td>40±3</td>
<td>21±3</td>
<td>49±3</td>
</tr>
<tr>
<td>1BD9</td>
<td>49±3</td>
<td>24±7</td>
<td>63±5</td>
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<td>1AC5</td>
<td>53±4</td>
<td>19±6</td>
<td>55±1</td>
</tr>
<tr>
<td>1AA1</td>
<td>42±4</td>
<td>16±2</td>
<td>24±1</td>
</tr>
<tr>
<td>1AE12</td>
<td>53±3</td>
<td>12±4</td>
<td>81±5</td>
</tr>
<tr>
<td>PBL</td>
<td>−12±10</td>
<td>−15±2</td>
<td>1±1</td>
</tr>
</tbody>
</table>

a LAK cell clones were used as effectors in a standard 4-hr 51Cr release assay against fresh (NK cell-resistant) tumor targets, PBL, and the cultured NK target K562.

Results are expressed as % specific lysis ± SEM. Effector-to-target cell ratio was 40:1. ND = not done. All LAK cell clone lysis is significant, P < .05, with the exception of that of 1AA1 vs. sarcoma #5 and colon cancer and 1AE12 vs. sarcomas #4 and 7 and colon cancer.

did not exceed the life-span of the unseparated population.

Lysis by LAK Cell Clones of Multiple Tumor Targets

To determine whether the lysis by LAK cells of multiple fresh tumor targets was maintained at the clonal level, we tested 10 LAK cell clones against a panel of targets including the autologous tumor, autologous PBL, 5 allogeneic sarcomas, an allogeneic colon cancer, and the cultured K562 target. Most effector-target combinations were tested in 3 separate assays with similar results. The results of a representative assay including 7 of the clones are presented in table 3. Each tumor target was susceptible to lysis by multiple clones. All clones lysed multiple tumor targets. None of the clones killed normal PBL. All fresh tumor targets were resistant to NK cell lysis. Quantitative heterogeneity in the LAK cell activity of the clones is evident by the comparison of a highly lytic clone (e.g., 1BH10) with a clone with lower LAK cell activity (e.g., 1AE12.) These results suggest that LAK cell clones, progeny of a single LAK cell, can recognize a determinant that is shared by multiple tumors, which is not present on normal PBL.

Subcloning of LAK Cell Clones

To confirm the validity of our observations, we subcloned 5 of the LAK cell clones. A total of 16 subclones were analyzed. The results of subcloning of 2 representative clones, 1BH10 with high LAK cell activity and 1AE12 with intermediate LAK cell activity, are presented. Subcloning plates were visually scored to determine the number of wells with growth as presented in text-figure 3 (1AE12 on day 13 after subcloning and 1BH10 on day 12). By regression analysis, the correlation between log(% negative wells) and number of cells per well was linear (1AE12: P = .0035; 1BH10: P = .0004). Subclones were expanded from plates that were seeded at 0.3 cell per well and 1 cell per well. Poisson analysis for the subcloning alone yielded the following probabilities that the subclones were derived from a single cell: 1AE12,

![Text-figure 3](https://academic.oup.com/jnci/article-abstract/75/1/67/941798/1AE12-SUBCLONE-SCORING)

![Text-figure 3](https://academic.oup.com/jnci/article-abstract/75/1/67/941798/1BH10-SUBCLONE-SCORING)

**Text-figure 3.**—Subclones derived from limiting dilution of LAK cell clones 1AE12 (A) and 1BH10 (B) were scored, as wells with growth, on days 13 and 12, respectively. The plot of log(%) negative wells versus the number of cells plated per well adheres to the Poisson distribution by linear regression analysis Poisson (P = .0035 and .0004, respectively).
TABLE 4.—Lysis by LAK cell subclones of multiple fresh human tumor targets

<table>
<thead>
<tr>
<th>Effectora</th>
<th>Autologous Sarcoma #1</th>
<th>Sarcoma #2</th>
<th>Sarcoma #3</th>
<th>Sarcoma #4</th>
<th>Allogeneic Colon Cancer</th>
<th>K562</th>
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<tbody>
<tr>
<td>LAK cell clone 1AE12</td>
<td>54±4</td>
<td>27±6</td>
<td>37±4</td>
<td>9±1</td>
<td>7±3</td>
<td>30±1</td>
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<tr>
<td>1AE12 subclones</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>τ0.3H5</td>
<td>47±4</td>
<td>41±4</td>
<td>38±1</td>
<td>13±1</td>
<td>2±4</td>
<td>34±1</td>
</tr>
<tr>
<td>τ1C4</td>
<td>60±10</td>
<td>24±8</td>
<td>36±4</td>
<td>6±3</td>
<td>0±3</td>
<td>31±1</td>
</tr>
<tr>
<td>τ1E6</td>
<td>54±1</td>
<td>14±11</td>
<td>28±4</td>
<td>15±1</td>
<td>-4±2</td>
<td>21±1</td>
</tr>
<tr>
<td>LAK cell clone 1BH10</td>
<td>58±3</td>
<td>32±1</td>
<td>44±3</td>
<td>20±1</td>
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<td>61±2</td>
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<tr>
<td>1BH10 subclones</td>
<td></td>
<td></td>
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</tr>
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<td>μ1C8</td>
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<td>43±2</td>
<td>16±3</td>
<td>ND</td>
<td>49±7</td>
</tr>
</tbody>
</table>

a LAK cell clones and their respective subclones were used as effectors in a standard 4-hr 51Cr release assay against fresh (NK cell-resistant) tumor targets and the cultured NK cell-sensitive target K562.

b Results are expressed as % specific lysis ± SEM. ND = not done. Effector-to-target cell ratio was 40:1 for all effector–target combinations with the exception of 10:1 for all effectors against colon cancer and 20:1 for β1C8 against all targets.

0.3 cell per well (97%) and 1 cell per well (93%); 1BH10, 0.3 cell per well (95%) and 1 cell per well (87%). With the 89% probability that the initial clones from the unseparated population derived from single cells, the overall probability that each of these subclones was the progeny of a single LAK cell was greater than or equal to 99%.

The in vitro growth of the subclones was similar to that of the clones and the unseparated population. The logarithmic rate of expansion and arrival at senescence paralleled that of the other cultures.

Lysis by LAK Cell Subclones of Multiple Tumor Targets

The LAK cell subclones were tested for lysis of tumor targets. The parent clones were tested in parallel, as presented in table 4. The subclones maintained the lytic characteristics of the parent clones. High LAK cell activity was manifested by the 1BH10 subclones, and in general lower lysis was evident for the 1AE12 subclones. All subclones killed multiple tumor targets. These results confirmed our observations with the LAK cell clones and support the existence of a shared determinant on tumor cells recognized by single LAK cell clones.

Phenotyping of LAK Cells

The phenotypes of the unseparated LAK cell population, 4 clones, and 1 subclone were determined with the use of monoclonal antibody staining and flow cytometry analysis.

Text-figure 4 displays the phenotypes of the unseparated population, the highly lytic clone 1BH10, and the clone with lower LAK cell activity (1AE12). The unseparated population was composed of Leu 4-positive cells (T-cells), with subsets that were brightly stained with Leu 2 (class I reactive, “cytotoxic-suppressor” phenotype) and Leu 3 (class II reactive, “helper” phenotype). These cells were Leu DR and TAC positive. Leu 7, the marker for NK cells, was negative. The clone with lower LAK cell activity (1AE12) was brightly stained with Leu 3 but not with Leu 2. A second clone with lower LAK cell activity (1AA1) had an identical phenotype (data not shown). The clone with high LAK cell activity (1BH10) stained brightly with Leu 2 but not with Leu 3. A second clone with high LAK cell activity (1BA6) and a subclone of 1BH10 (1B1G10) had the same IF profile (data not shown).

Thus LAK clone 1AE12 was Leu 2-3+4+r DR+Tac+, and clone 1BH10 was Leu 2+3- 4+7-dr+ Tac+. These results demonstrate that LAK cell activity is manifested both by cells with the phenotype of class I reactive and class II reactive cells.

DISCUSSION

This report presents our results of the cloning of a population of LAK cells generated by the incubation of PBL in IL-2. We have previously reported that unseparated populations of LAK cells can kill multiple fresh human tumor targets and spare normal tissue (1-3). In a recent study 36 of 41 fresh tumor target cells with varied histologies were shown to be susceptible to LAK cell lysis (4). The purpose of this current study was to determine whether the killing of multiple tumors was a manifestation of the polyspecificity of individual LAK cells or was due to the presence of subpopulations of LAK cells with restricted specificity.

The distinguishing feature of LAK cell lysis is the ability to kill fresh, noncultured tumor targets. Other methods of activation of human non-CTL lytic cell systems have been described, including allosensitization, autologous mixed lymphocyte reaction, mixed lymphocyte tumor culture, interferon, lectins, bacterial extracts, and xenographic serum (10-28). These may be variants of LAK cell generation, since most involve either culture in...
TEXT-Figure 4.—The IF profiles of cell surface phenotype demonstrate that LAK cells bear determinants reactive with monoclonal antibodies Leu 4, Leu DR, and anti-Tac. The marker for NK cells (Leu 7) did not stain LAK cells. The unseparated population contained subpopulations that stained brightly with Leu 2 or Leu 3. The LAK cell clone with intermediate lysis (IAE12) stained brightly with Leu 3; the highly lytic clone (IBH10) stained brightly with Leu 2. Thus clone IAE12 was Leu $2^+3^+4^+7^+\text{DR}^\text{+Tac}^+$ and clone IBH10 was Leu $2^+3^+4^+7^+\text{DR}^\text{+Tac}^+$. 

IL-2-containing media or processes that are known or are likely to result in IL-2 production. Most of these systems have been tested against cultured tumor targets and have not been analyzed against noncultured tumors. We have, however, shown that activated killer cells generated by allosensitization and lectin incubation can lyse fresh human tumor targets and, in addition, lyse virtually all cultured cell lines and mitogen-activated lymphoblasts ($18, 19, 24, 25$). We have demonstrated that IL-2 alone is sufficient for the generation of LAK cells ($32$) and that recombinant IL-2 will generate a population of LAK cells with broad lytic capacity for fresh tumor targets ($4, 33$).

The principal targets for the analysis described in this paper are fresh, noncultured tumors. We have included the cultured NK cell-sensitive target K562 in most assays to demonstrate that the fresh tumor targets were not lysed by the nonactivated NK cells present in PBL. PBL targets were included as a readily available normal tissue to confirm the sparing of normal tissue by LAK cell lysis, as has previously been shown with normal lung and liver cells ($3$).

Cloning and subcloning require long-term in vitro maintenance of rapidly expanding cultures. Although IL-2 alone generates LAK cells, the sustained growth of LAK cells appears to require that both PHA and feeder cells are added to cultures ($4$). This experience is in agreement with the analyses of augmented growth of T-cells by PHA and feeder cells reported by other investigators ($13, 34-38$). The total in vitro life-span from the initial IL-2 activation of PBL to the senescence of the LAK cell culture was $2.5$ months. The process of cloning and subcloning did not appear consistently to extend or shorten the maximum in vitro life-span of the cultures. The estimated number of population doublings for the cultures was $50-60$. This observation is in accord with the conclusions of others regarding the in vitro growth of nontransformed T-cells ($13, 39$).

Further analysis is needed to delineate the role of PHA in maintenance of LAK cell cytotoxicity. Culture of LAK cell clones in the absence of PHA has led to concomitant diminished proliferation, loss of IL-2 receptors, and loss of cytotoxicity (Rayner AA: Unpublished data). In the current series of experiments, cloned LAK cells were washed to eliminate PHA prior to testing, and PHA was not present in the medium used for the cytotoxicity assays. We have demonstrated that IL-2 alone (in the absence of PHA), including recombinant IL-2, generates LAK cells from nonlytic precursors ($4, 32, 33$). Other investigators have correlated diminished proliferation of T-cells with absence or loss of IL-2 receptors when cells are cultured in the absence of PHA and have observed the induction ($36$) or restoration ($37$) of growth and IL-2 receptors when culture media were supplemented with PHA. Further studies will be necessary to determine whether loss of LAK cytotoxicity is a function of altered growth or a manifestation of requirement for an additional signal or whether PHA plays a direct role in the cytotoxicity of these clones by a cross-linking mechanism similar to that of lectin-dependent cytotoxicity.

Cloning by limiting dilution produced wells with growth that conformed to the Poisson distribution when subjected to linear regression analysis. This validated our use of Poisson statistics to verify the high probability for derivation of our clones from a single LAK cell. Similar analysis of the subclones confirmed a greater than or equal to $99\%$ probability that our cultures were true clones.
The initial cytotoxicity testing of clones demonstrated that most, if not all, of the clonogenic cells in the unseparated LAK cell population were lytic cells. These assays also identified LAK cell clones with consistently high or with consistently lower LAK activity. When LAK cell clones were tested against a panel of targets, the polyspecificity for tumor targets was maintained by the progeny of a single LAK cell. All clones killed multiple tumor targets, sparing PBL. Each tumor target was killed by multiple LAK cell clones. The corroboration of these findings by the LAK cell subclones adds to the reliability of these observations. These data suggest the existence of a common recognition determinant on tumor cells that is not present on PBL. The presence of this determinant is further supported by findings in cold target inhibition assays that demonstrate inhibition of LAK cell-mediated tumor lysis by various tumors, without inhibition by normal PBL (Grimm EA: Unpublished data).

Phenotyping of the unseparated population of LAK cells demonstrated that it was composed of T-cells that stained either with Leu 2 or Leu 5 monoclonal antibody. The LAK cell clones were stained with either Leu 2 or Leu 3. Cytolytic capacity of class II reactive ("helper" phenotype) T-cells has been reported by others (13, 40, 41). The LAK cells were not stained by Leu 7, which binds to NK cells. The LAK cells did stain with Leu DR (Ia analogue) and anti-Tac (IL-2 receptor).

The successful application of cloning techniques to human non-CTL lytic systems has now been reported by multiple investigators (13, 15, 16, 34, 42). The methods were similar, utilizing feeder cells from a variety of sources and employing supernatants of PHA-stimulated PBL as a source of IL-2, most often with additional PHA in the media of the clone cultures. Allosensitization was performed prior to cloning by some (13, 15, 17), whereas others enriched for large granular lymphocytes by Percoll fractionation (34) or anti-T-cell reagents (43). We have previously reported broad lysis of fresh tumor targets by effectors generated in allosensitization (18, 19) and by large granular lymphocyte-enriched populations activated in IL-2 (3, 5). All investigators reported lysis of multiple cultured tumor targets. The predominant use of NK cell-sensitive, cultured tumor targets, however, makes it difficult to compare most of these results with our own observations. In the absence of cytotoxicity data for fresh tumor targets, the methodologies and cell surface phenotypes suggest that these analyses included clones likely to represent LAK cell clones (13, 16, 34) as well as true NK cell clones (15, 43).

The biologic significance of LAK cells in vivo immune response is, at present, a matter of conjecture. One possible biologic role of LAK cells is that of a recruited polyspecific effector cell population. An immune response that induces high local concentrations of IL-2 could result in the generation of LAK cells that would kill altered cells but not normal tissue. We have reported that the systemic adoptive transfer of LAK cells in mice bearing established syngeneic pulmonary metastases can reduce metastases and prolong survival of the mice (44, 45). We are currently exploring the use of LAK cells for the adoptive immunotherapy of human tumors.

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