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## A MODEL FOR THE DIFFERENTIATION OF HUMAN NATURAL KILLER CELLS

Studies on the In Vitro Activation of Leu-11<sup>+</sup> Granular Lymphocytes  
With a Natural Killer-sensitive Tumor Cell, K562

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Natural killer (NK)<sup>1</sup> cells are a heterogeneous population of lymphoid cells from an unimmunized host that are able to lyse certain tumor cells and virally infected cells through a mechanism unrestricted by the major histocompatibility complex (MHC). Human lymphocytes with NK activity can be highly enriched in the low density fractions of discontinuous Percoll gradients (1). Morphological examination of this low density population demonstrates that the majority of these cells are large granular lymphocytes (LGL) (1). Recently (2–5), several murine monoclonal antibodies, including anti-Leu-11, B73.1, 3G8, and VEP 13, have been produced that react with essentially all human NK cells, but not T cells, B cells, or monocytes. Anti-Leu-11 and related mAb react with an antigen associated with the Fc receptor for IgG present on NK cells and neutrophils, and can specifically inhibit Fc receptor-mediated functions (2, 3, 5).

Lymphoblasts with the characteristics of NK cells have been identified in mixed lymphocyte response cultures using autologous and allogeneic stimulators (6–13). Recently (6), we have examined the phenotypic, functional, and morphological characteristics of the lymphoblasts generated in a mixed lymphocyte/tumor cell response (MLTR) culture using peripheral blood mononuclear cells as responders and an allogeneic B cell line as the stimulator. In these cultures, the predominant cell type was a CD3<sup>+</sup> T lymphoblast. Typically, only 5–15% of the total lymphoblasts expressed the NK cell-associated antigen, Leu-11. Antigen-specific cytotoxicity against the stimulating B cell line was mediated by a typical CD3<sup>+</sup> cytotoxic T lymphoblast. In contrast, the majority of the cells mediating non-MHC-restricted cytotoxicity against the NK-sensitive tumor K562 expressed the Leu-11 antigen and lacked pan T cell-associated markers (6). The Leu-11<sup>+</sup> cells isolated from these MLTR cultures demonstrate enhanced cytotoxic activity against NK-sensitive and -insensitive tumor cells, and blast morphology; in addition, they express antigens associated with cellular activation, e.g., class II MHC antigens and transferrin receptor (6). Interferon and interleukin 2 (IL-2) efficiently enhance NK cell-mediated cytotoxicity (14–21), and cell

<sup>1</sup> *Abbreviations used in this paper:* CR<sub>3</sub>, complement receptor type 3; E/T, effector/target; FACS, fluorescence-activated cell sorter; FITC, fluorescein isothiocyanate; IL-2, interleukin 2; LGL, large granular lymphocytes; mAb, monoclonal antibody; MHC, major histocompatibility complex; MLTR, mixed lymphocyte/tumor response; NK, natural killer; PE, phycoerythrin; PHA, phytohemagglutinin.

lines with NK cell characteristics have been established using IL-2 as growth factor (22–25). Since interferons and IL-2 are present in high concentration in mixed lymphocyte response (MLR) cultures, it seemed likely that activation of the Leu-11<sup>+</sup> cells resulted from a response to these soluble factors. However, it was unresolved whether NK cells in the MLTR could directly respond against the stimulator.

In the present experiments, we have questioned whether NK cells can be activated by direct stimulation with the NK-sensitive tumor cell line, K562. Using highly purified populations of NK (Leu-11<sup>+</sup>) and T (Leu-11<sup>-</sup>) lymphocytes, we observed that Leu-11<sup>+</sup> cells were activated when cocultured with mitomycin C-treated K562 cells. This activation process resulted in proliferation, enhanced cytotoxic activity, and expression of activation antigens, such as transferrin receptor and class II MHC antigens. The activation was a direct consequence of interactions between Leu-11<sup>+</sup> cells and K562. Exogenous growth factors or accessory cells were not required. Previously (2), we demonstrated that Leu-11<sup>+</sup> lymphocytes could be divided into discrete subpopulations on the basis of Leu-7 antigen expression. We now report that expression of the Leu-7 antigen can be induced in the Leu-7<sup>+</sup>11<sup>+</sup> subset by activation with K562. In contrast, stimulation of the Leu-7<sup>+</sup>11<sup>+</sup> cells with K562 did not affect the antigenic phenotype. These data establish a developmental relationship between the Leu-7<sup>-</sup>11<sup>+</sup> and Leu-7<sup>+</sup>11<sup>+</sup> cells and are consistent with the possibility that Leu-7 is a differentiation antigen that appears late in the development of NK cells.

### Materials and Methods

*Preparation of Human Peripheral Blood Leukocytes.* Human peripheral blood from randomly chosen normal donors was obtained from the American Red Cross, San Jose, CA or the Stanford Blood Center, Palo Alto, CA. Mononuclear cells were isolated using Ficoll/Hypaque (Pharmacia Fine Chemicals, Piscataway, NJ) by standard methods. Cells were washed extensively in phosphate-buffered saline (0.1 M phosphate, pH 7.3).

*Enrichment of LGL.* Monocytes were depleted from the peripheral blood mononuclear cells by adherence to plastic tissue culture flasks (26). B lymphocytes and residual adherent cells were removed by passing the cells through nylon wool (26). Nylon wool–nonadherent cells were layered onto a cushion of 43.5% Percoll, centrifuged for 50 min at 300 g, and the low density fraction was harvested from the gradient interface. We have demonstrated (26), using this procedure, that essentially all lymphocytes with NK activity are contained in the low density fraction. Typically, ~30–60% of the low density lymphocytes expressed the NK cell-associated antigen, Leu-11, whereas ~40–70% expressed the pan T cell antigen, CD3, depending on the individual donor.

*Monoclonal Antibodies.* All antibodies were prepared by the Becton Dickinson Monoclonal Center, Inc. Anti-Tac (IL-2 receptor) mAb was generously provided by Dr. T. Waldmann, National Institutes of Health (27). Anti-Leu-4 mAb recognizes the pan T cell antigen CD3. Anti-Leu-11 reacts with an antigen (CD16) associated with the Fc receptor for IgG present on essentially all NK cells and neutrophils (2, 5). Anti-Leu-2a and anti-Leu-3a react with the CD8 and CD4 antigens, respectively. Anti-Leu-7 reacts with a subset of NK cells (2, 28). Anti-Leu-15 reacts with an antigen associated with the complement receptor type 3 (CR<sub>3</sub>) (L. Lanier and G. Ross, unpublished observation). Anti-Leu-M3 reacts with an antigen exclusively present on monocytes. The CD nomenclature for human differentiation antigens, proposed by the International Workshop on Human Leukocyte Differentiation Antigens (29) and approved by the World Health Organization, will be used to describe the T cell differentiation antigens discussed in this paper.

*Immunofluorescence and Flow Cytometry.* Immunofluorescent staining procedures have

been described previously (2, 30). Fluorochrome-conjugated, isotype-matched mAb that did not specifically react with human cells were used to control for nonspecific binding. Immunofluorescence was measured using a fluorescence-activated cell sorter (FACS) (FACS 440; Becton Dickinson Immunocytometry Systems, Mountain View, CA). Methods of flow cytometry have been described in detail elsewhere (2, 30).

*Tumor Cells.* All tumor cell lines were obtained from the American Type Culture Collection, Rockville, MD and were tested monthly to ensure against mycoplasma contamination.

*In Vitro Stimulation of Lymphocytes Subsets.* Lymphocytes were cocultured with mitomycin C-treated (80  $\mu\text{g}/\text{ml}$ , 1 h) tumor cells in RPMI 1640 (Irvine Scientific, Santa Ana, CA) supplemented with 5% heat-inactivated horse serum (JR Scientific, Woodland, CA), 1 mM glutamine (Gibco Laboratories, Grand Island, NY), and antibiotics (complete culture medium). Responder cells in complete culture medium ( $0.5\text{--}5 \times 10^6/\text{ml}$ ) were mixed with stimulator cells ( $0.1\text{--}1.0 \times 10^6/\text{ml}$ ) to achieve a ratio of 5:1. Cells were placed into 96-well round-bottom or 24-well plastic tissue culture plates (Falcon Plastics, Oxnard, CA). Lymphocytes ( $1 \times 10^6/\text{ml}$ ) in complete culture medium were stimulated with phytohemagglutinin (PHA-M) (Gibco Laboratories) at a concentration of 2% (wt/vol). Lymphocyte proliferation was measured by incorporation of [ $^3\text{H}$ ]thymidine. Cultures were pulsed overnight with 1  $\mu\text{Ci}$  [ $^3\text{H}$ ]thymidine (Amersham Corp., Arlington Heights, IL), and the cells harvested using a PHD Cell Harvester (Cambridge Technology, Inc., Cambridge, MA). Samples were placed in vials containing Complete Counting Cocktail 3a20 (Research Products International Corp., Mt. Prospect, IL) and the radioactivity determined using an LS 7800 liquid scintillation counter (Beckman Instruments, Inc., Fullerton, CA). The mean of triplicate samples was used for all statistical evaluation.

*Cytotoxicity Assays.* Tumor cells were labeled with  $^{51}\text{Cr}$  and were used as targets in a 4-h radioisotope release cytotoxicity assay, as described previously (2).

*Morphology.* Cells were cytocentrifuged onto ethanol-cleaned glass slides. The specimens were fixed in absolute methanol for 10 min, air dried, stained in a 10% aqueous solution of Giemsa for 10 min, and washed in distilled water.

## Results

*Stimulation of Leu-11<sup>-</sup> and Leu-11<sup>+</sup> Lymphocytes With K562.* Peripheral blood lymphocytes from randomly selected normal individuals, depleted of monocytes by adherence, were stained with fluorescein isothiocyanate (FITC)-conjugated anti-Leu-11a mAb. ~10–20% of the lymphocytes were antigen positive, depending on the donor. Lymphocytes were sorted into Leu-11<sup>-</sup> and Leu-11<sup>+</sup> fractions using a FACS. The isolated subpopulations, >90% pure as determined by reanalysis, were cocultured with an NK-sensitive tumor cell line, K562, or a relatively NK-insensitive B cell line, CCRF-SB. The stimulator cell lines were treated with mitomycin C to prevent cell division. Proliferation by the responder cells was measured on day 6 by [ $^3\text{H}$ ]thymidine incorporation. Leu-11<sup>+</sup> cells significantly proliferated in response to coculture with K562, but usually not CCRF-SB (Table I). In contrast, Leu-11<sup>-</sup> lymphocytes strongly proliferated in response to the B cell line CCRF-SB, but reacted only weakly against K562. This preferential activation of Leu-11<sup>+</sup> cells against K562 was observed in all donors, although the magnitude of the response varied (Table I). Kinetic studies revealed that the proliferative response of both the T lymphocytes against CCRF-SB and the NK cells against K562 peaked on day 5 of culture (Fig. 1).

Cells harvested after 6 d of coculture with K562 were examined by flow cytometry for light scatter characteristics and expression of surface differentiation antigens. As shown in Fig. 2, lymphocytes that expressed the Leu-11 antigen before coculture with K562 retained Leu-11 expression after 6 d. Similarly, all

TABLE I  
Proliferation of *Leu-11<sup>+</sup>* and *Leu-11<sup>-</sup>* Lymphocytes Cocultured With K562

Exp.	Responder	Stimulator	[ <sup>3</sup> H]thymidine incorporation <i>cpm</i>	Stimulation index
1	<i>Leu-11<sup>-</sup></i>	—	431 ± 109	—
	<i>Leu-11<sup>+</sup></i>	—	321 ± 57	—
	<i>Leu-11<sup>-</sup></i>	K562	1,549 ± 312*	3.6
	<i>Leu-11<sup>+</sup></i>	K562	10,523 ± 814	32.8
	<i>Leu-11<sup>-</sup></i>	CCRF-SB	5,844 ± 114	13.6
	<i>Leu-11<sup>+</sup></i>	CCRF-SB	143 ± 72	0
2	<i>Leu-11<sup>-</sup></i>	—	580 ± 150	—
	<i>Leu-11<sup>+</sup></i>	—	610 ± 56	—
	<i>Leu-11<sup>-</sup></i>	K562	1,600 ± 200	2.7
	<i>Leu-11<sup>+</sup></i>	K562	16,120 ± 240	26.4
3	<i>Leu-11<sup>-</sup></i>	—	820 ± 110	—
	<i>Leu-11<sup>+</sup></i>	—	341 ± 120	—
	<i>Leu-11<sup>-</sup></i>	K562	3,280 ± 260	4.0
	<i>Leu-11<sup>+</sup></i>	K562	6,138 ± 220	18.0

Peripheral blood mononuclear cells were stained with FITC anti-*Leu-11a* mAb, then separated into *Leu-11<sup>-</sup>* and *Leu-11<sup>+</sup>* lymphocyte subpopulations using a FACS. Reanalysis of the sorted cells indicated >95% purity. Lymphocytes were cocultured with or without mitomycin C-treated K562 or CCRF-SB tumor cells, as indicated. The culture wells were pulsed overnight with 1  $\mu$ Ci [<sup>3</sup>H]-thymidine and harvested after 6 d culture.

\* Background thymidine incorporation by the mitomycin C-treated K562 or CCRF-SB tumor cells (tumor inactivation control) was subtracted from the raw counts to determine the specific incorporation by the effector cell population. Stimulation index: (cpm with stimulator)/(cpm without stimulator).

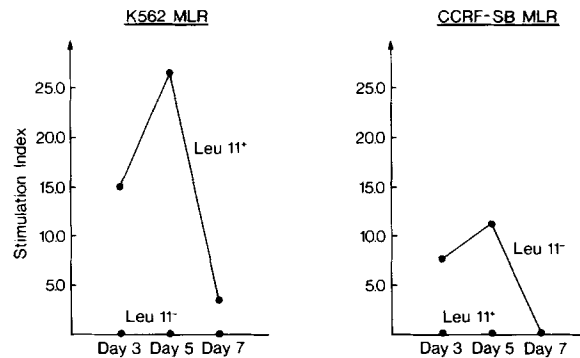


FIGURE 1. Kinetics of proliferative response. Peripheral blood mononuclear cells were stained with FITC anti-*Leu-11a* mAb and separated into *Leu-11<sup>-</sup>* and *Leu-11<sup>+</sup>* lymphocyte subpopulations using a FACS. Reanalysis indicated >95% purity. >99% of the sorted cells were lymphocytes by morphological criteria. *Leu-11<sup>-</sup>* and *Leu-11<sup>+</sup>* lymphocytes were cocultured with mitomycin C-treated K562 (K562 MLR) or CCRF-SB (CCRF-SB MLR) cells in 96-well microtiter plates. Cultures were pulsed overnight with [<sup>3</sup>H]thymidine and harvested on days 3, 5, and 7. The stimulation index is defined as: (cpm with stimulator)/(cpm without stimulator).

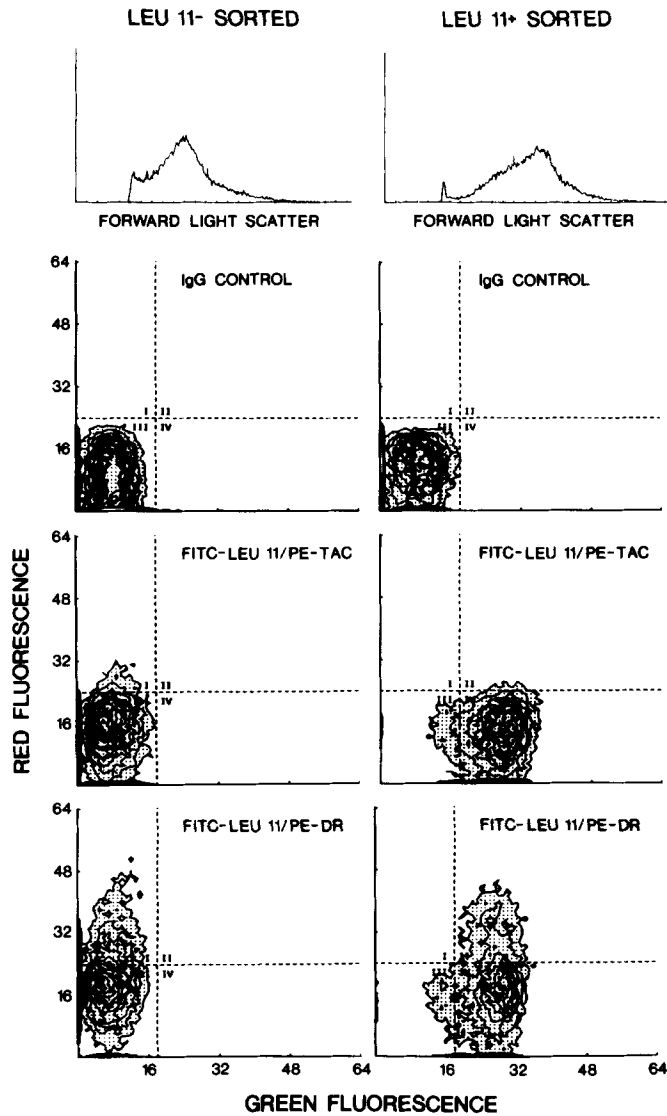


FIGURE 2. Blastogenesis and expression of activation antigens on lymphocyte subsets cocultured with K562. Peripheral blood mononuclear cells were stained with FITC anti-Leu-11a mAb and separated into Leu-11<sup>-</sup> and Leu-11<sup>+</sup> lymphocyte subpopulations using a FACS. Reanalysis indicated >90% purity. >99% of the sorted cells were lymphocytes by morphological criteria. Leu-11<sup>-</sup> (left) and Leu-11<sup>+</sup> (right) lymphocytes were cocultured with mitomycin C-treated K562 in 24-well culture plates. After 6 d, cells were harvested and stained with: FITC IgG and PE IgG control antibodies; FITC anti-Leu-11a and PE anti-Tac; or FITC anti-Leu-11a and PE anti-HLA-DR. In the upper panels, histograms displaying the forward angle light scatter are presented (x axis, 256 channels, linear scale; y axis, relative number of cells). Forward angle light scatter measurements essentially reflect cell size for lymphoid cells (31). In the lower panels, correlated measurements of green (x axis, log scale) and red (y axis, log scale) fluorescence are displayed as two-dimensional contour plots (2, 30). Based on the control sample, the contour plots were divided into quadrants: I, cells with red fluorescence only; II, cells with green and red fluorescence; III, unstained cells; and IV, cells with green fluorescence only. The number of cells in each area was integrated and the percentage of the total cells was determined. In the control samples, <5% of the lymphocytes were nonspecifically stained (i.e., present in quadrants I, II, and IV). In the population of Leu-11<sup>-</sup> cells cocultured with K562 (left), <5% expressed the Leu-11 antigen. 31.7% of the cells were Leu-11<sup>-</sup>, DR<sup>+</sup> and <5% were Leu-11<sup>-</sup>, Tac<sup>+</sup>. In contrast, after stimulation of the Leu-11<sup>+</sup> cells with K562, ~85–90% of these cells expressed Leu-11 antigen and 25.9% coexpressed Leu-11 and DR antigen. <5% of the Leu-11<sup>+</sup> lymphocytes coexpressed Leu-11 and Tac antigen. (It should be noted that the density of Leu-11 antigen decreases after activation and may lead to underestimation of the percentage of dimly stained Leu-11<sup>+</sup> cells).

lymphocytes that lacked Leu-11 antigen before coculture with K562 were Leu-11<sup>-</sup> after 6 d. Thus, there was no evidence for acquisition or loss of this antigen as a consequence of stimulation with K562. A proportion of the Leu-11<sup>+</sup> cells coexpressed HLA-DR antigen (25% in Fig. 2) and transferrin receptor (not shown) after coculture with K562. Before culture, <1% of Leu-11<sup>+</sup> cells coexpressed HLA-DR or transferrin receptor, indicating that class II MHC antigens and transferrin receptor were induced as a consequence of activation. Further evidence for activation of the Leu-11<sup>+</sup> cells was obtained by examining the forward angle light scatter properties of these cells. For lymphoid cells, light scatter is essentially proportional to cell size (31). In contrast to the Leu-11<sup>-</sup> cells that proliferated only weakly in response to coculture with K562, a majority of the Leu-11<sup>+</sup> cells demonstrated light scatter characteristics similar to mitogen-activated lymphoblasts (Fig. 2). This was confirmed by microscopic examination. A majority of the K562-activated Leu-11<sup>+</sup> cells were lymphoblasts with abundant cytoplasm and azurophilic granules (not shown). IL-2 receptor-associated antigen, Tac, was not detected on these Leu-11<sup>+</sup> lymphoblasts (Fig. 2). Similar results were obtained in three independent experiments.

A more extensive antigenic profile of the K562-activated Leu-11<sup>+</sup> lymphoblasts is presented in Fig. 3. A proportion of the Leu-11<sup>+</sup> lymphoblasts coexpressed

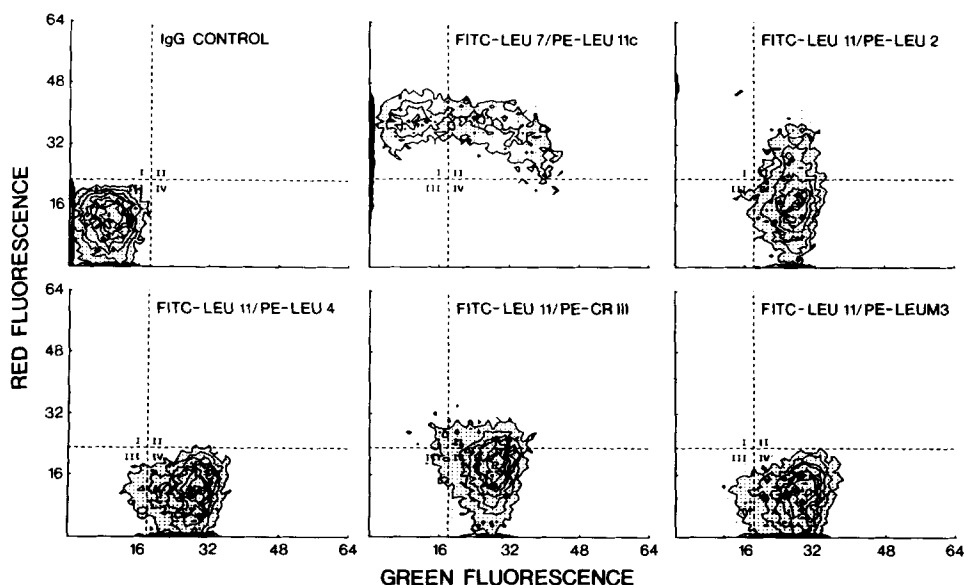


FIGURE 3. Antigenic phenotype of Leu-11<sup>+</sup> lymphocytes after coculture with K562. Purified Leu-11<sup>+</sup> lymphocytes were cocultured with mitomycin C-treated K562 cells, as described in Fig. 2. After 6 d, viable cells were recovered and stained with: FITC IgG and PE IgG control antibodies; FITC anti-Leu-7 and PE anti-Leu-11c; FITC anti-Leu-11a and PE anti-Leu-2; FITC anti-Leu-11a and PE anti-Leu-4; FITC anti-Leu-11a and PE anti-CR<sub>3</sub>(Leu-15); and FITC anti-Leu-11a and PE anti-Leu-M3. Data are presented as described in Fig. 3. Quantitation of the results revealed: 43.7% Leu-11<sup>+</sup>7<sup>+</sup>; 43.3% Leu-11<sup>+</sup>7<sup>-</sup>; <5% Leu-11<sup>-</sup>7<sup>+</sup>; 26.3% Leu-11<sup>+</sup>2<sup>+</sup>; 56.8% Leu-11<sup>+</sup>2<sup>-</sup>; 8.6% Leu-11<sup>-</sup>2<sup>+</sup>; <5% Leu-11<sup>-</sup>2<sup>-</sup>; <5% Leu-11<sup>+</sup>4<sup>+</sup>; 83.5% Leu-11<sup>+</sup>4<sup>-</sup>; <5% Leu-11<sup>-</sup>4<sup>+</sup>; 17.5% Leu-11<sup>+</sup>CR<sub>3</sub><sup>+</sup>; 67.6% Leu-11<sup>+</sup>CR<sub>3</sub><sup>-</sup>; 6.3% Leu-11<sup>-</sup>CR<sub>3</sub><sup>+</sup>; <5% Leu-11<sup>-</sup>M3<sup>+</sup>; 85.3% Leu-11<sup>+</sup>M3<sup>-</sup>; <5% Leu-11<sup>-</sup>M3<sup>+</sup>. >95% of the cells in the IgG control sample were unstained (quadrant III).

Leu-2 (CD8), Leu-7, and Leu-15 (CR<sub>3</sub> associated) antigens. These antigens are expressed on subpopulations of resting Leu-11<sup>+</sup> cells isolated from fresh blood (2, 32). The only notable difference between the freshly isolated Leu-11<sup>+</sup> cells and the activated Leu-11<sup>+</sup> lymphoblasts was that the cell surface density of Leu-15 apparently decreases during culture. Similarly, we observed that the density of Leu-11 antigen decreases after activation. Leu-M3, an antigen expressed on most monocytes, and Leu-4 (CD3), an antigen associated with the T cell antigen receptor complex, were not detected on the K562-activated Leu-11<sup>+</sup> lymphoblasts (Fig. 3).

The cytotoxic activity of the Leu-11<sup>-</sup> and Leu-11<sup>+</sup> cells was determined after coculture with or without mitomycin C-treated K562 cells for 6 d. Leu-11<sup>-</sup> lymphocytes cocultured with K562 usually demonstrated minimal cytotoxicity against K562 (Fig. 4), although low levels of cytotoxicity were occasionally observed in some individuals. Lymphoblasts derived from cocultures of Leu-11<sup>+</sup> lymphocytes and K562 were significantly more cytotoxic against K562 than Leu-11<sup>+</sup> cells cultured for 6 d without K562 (Fig. 4). Moreover, the K562-activated Leu-11<sup>+</sup> lymphoblasts were highly cytotoxic at low effector-to-target (E/T) ratios for a broad range of tumor cell targets, including U937, CEM, HSB-2, CCRF-SB, HUT-78, and K562. (Fig. 5).

*Stimulation of Subsets Identified by Expression of the Leu-7 and Leu-11 Antigens.* Peripheral blood lymphocytes can be divided into at least four distinct subpopulations based on the expression of the Leu-7 and Leu-11 surface antigens; i.e., Leu-7<sup>-</sup>11<sup>+</sup>, Leu-7<sup>+</sup>11<sup>+</sup>, Leu-7<sup>+</sup>11<sup>-</sup>, and Leu-7<sup>-</sup>11<sup>-</sup> (2). Lymphocytes, enriched for LGL by centrifugation on Percoll gradients, were stained with FITC anti-Leu-11a and phycoerythrin (PE)-avidin/biotin anti-Leu-7. The four subpopulations were separated using two-color FACS procedures and were cocultured

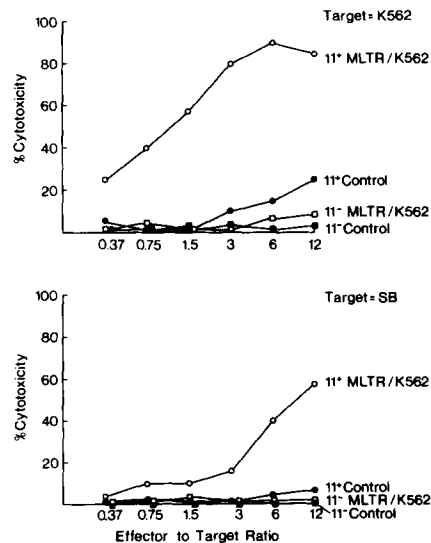


FIGURE 4. Cytotoxic activity of Leu-11<sup>-</sup> and Leu-11<sup>+</sup> lymphocytes after coculture with K562. Leu-11<sup>-</sup> and Leu-11<sup>+</sup> lymphocytes were cocultured with (*MLTR/K562*) or without (*control*) mitomycin C-treated K562 cells, as described in Fig. 2. After 6 d, viable cells were isolated and tested for cytotoxic activity against K562 and CCRF-SB in a 4 h radioisotope release assay.



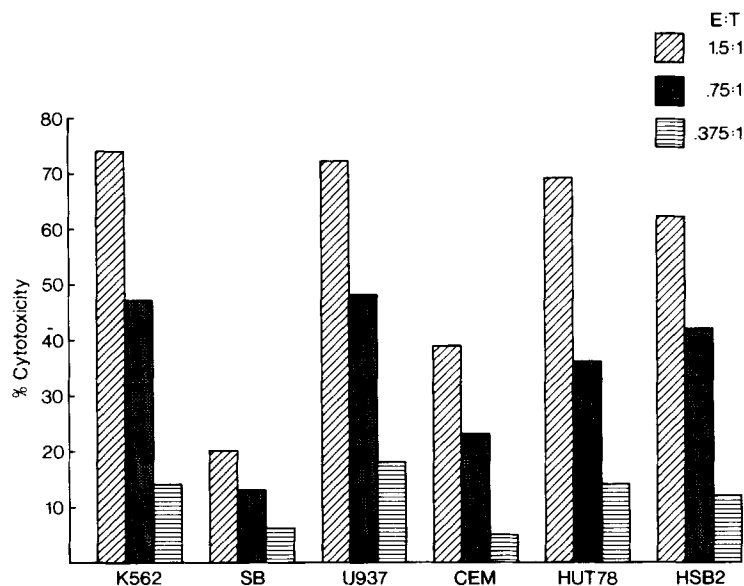


FIGURE 5. Cytotoxicity of activated Leu-11<sup>+</sup> lymphocytes. Leu-11<sup>+</sup> lymphocytes were cocultured with mitomycin C-treated K562 cells for 6 d, as described in Fig. 2. Viable cells were isolated and tested for cytotoxic activity against K562, CCRF-SB, U937, CEM, HUT-78, and HSB-2 tumor cell targets in a 4 h radioisotope release assay. Unstimulated Leu-11<sup>+</sup> lymphocytes and Leu-11<sup>-</sup> lymphocytes did not lyse (<5%) any of these targets at comparable E/T ratios (not shown).

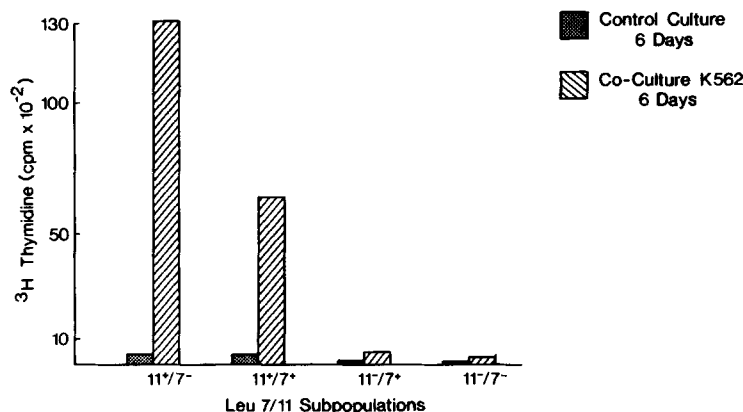


FIGURE 6. Proliferation of lymphocytes expressing the Leu-7 and/or Leu-11 antigens cocultured with K562. Nylon wool-nonadherent peripheral blood lymphocytes, enriched for NK cells using Percoll (Materials and Methods), were stained with FITC anti-Leu-11a and PE-avidin/biotin antiLeu-7. The resulting four subsets were separated by two-color FACS sorting. Reanalysis of the isolated populations indicated that all subsets were >95% pure. The cells were cocultured with or without mitomycin C-treated K562 cells in 96-well round-bottom plates for 6 d. Cultures were pulsed overnight with 1  $\mu$ Ci [<sup>3</sup>H]thymidine and harvested.

with mitomycin C-treated K562 cells. After 6 d of culture, proliferation was measured by [<sup>3</sup>H]thymidine incorporation (Fig. 6). Both the Leu-7<sup>+</sup>11<sup>+</sup> and Leu-7<sup>-</sup>11<sup>+</sup> proliferated in response to K562, with the latter population consistently

demonstrating significantly higher levels of proliferation (2–10-fold higher in three experiments, depending on the donor). The Leu-7<sup>+</sup>11<sup>-</sup> and Leu-7<sup>-</sup>11<sup>-</sup> lymphocytes were weakly stimulated by K562, although significant proliferation was observed in some donors (e.g., Table II, experiment 2). The anti-Leu-7 and anti-Leu-11 antibodies were not mitogenic for these cell populations, as demonstrated by the lack of proliferation in the cultures without K562 (Fig. 6). Furthermore, when Percoll gradient-enriched LGL were stained with anti-Leu-7 and anti-Leu-11 antibodies before cocultured with mitomycin C-treated K562 cells, the stimulation index was essentially identical to the response of LGL not stained with these antibodies. Therefore, it is unlikely that using these mAb for isolation of these subpopulations significantly influenced their response.

Analysis of the cytotoxic activity of the subpopulations after culture with K562 indicated that the Leu-7<sup>-</sup>11<sup>+</sup> and Leu-7<sup>+</sup>11<sup>+</sup> cells were both potent cytotoxic effectors. The Leu-7<sup>-</sup>11<sup>-</sup> and Leu-7<sup>+</sup>11<sup>-</sup> lymphocytes cocultured with K562 demonstrated minimal cytotoxic activity against K562 (Fig. 7). Previously (2), we demonstrated that the anti-Leu-7 and anti-Leu-11 antibodies do not affect the cytotoxic function mediated by NK cells.

With respect to antigenic phenotype, we consistently observed that a significant proportion (20–40% in three independent experiments, depending on the donor; 37.7% in Fig. 8) of the Leu-7<sup>-</sup>11<sup>+</sup> cells acquired expression of Leu-7 antigen after stimulation with K562 (Fig. 8). It is unlikely that the appearance of the Leu-7<sup>+</sup>11<sup>+</sup> cells in the Leu-7<sup>-</sup>11<sup>+</sup> population after stimulation with K562 resulted

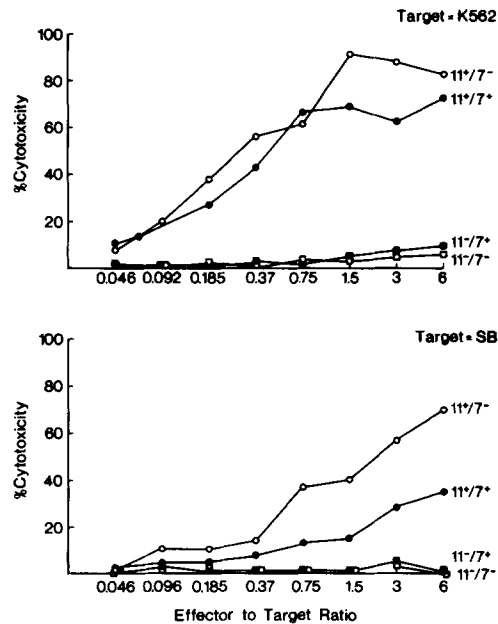


FIGURE 7. Cytotoxic activity of lymphocytes expressing the Leu-7 and/or Leu-11 antigens cocultured with K562. Lymphocyte subsets expressing the Leu-7 and/or Leu-11 antigens were isolated by two-color FACS sorting and cocultured with mitomycin C-treated K562 cells, as described in Fig. 6. After 6 d, the viable cells were harvested and tested for cytotoxic activity against K562 and CCRF-SB using a 4 h radioisotope release assay.

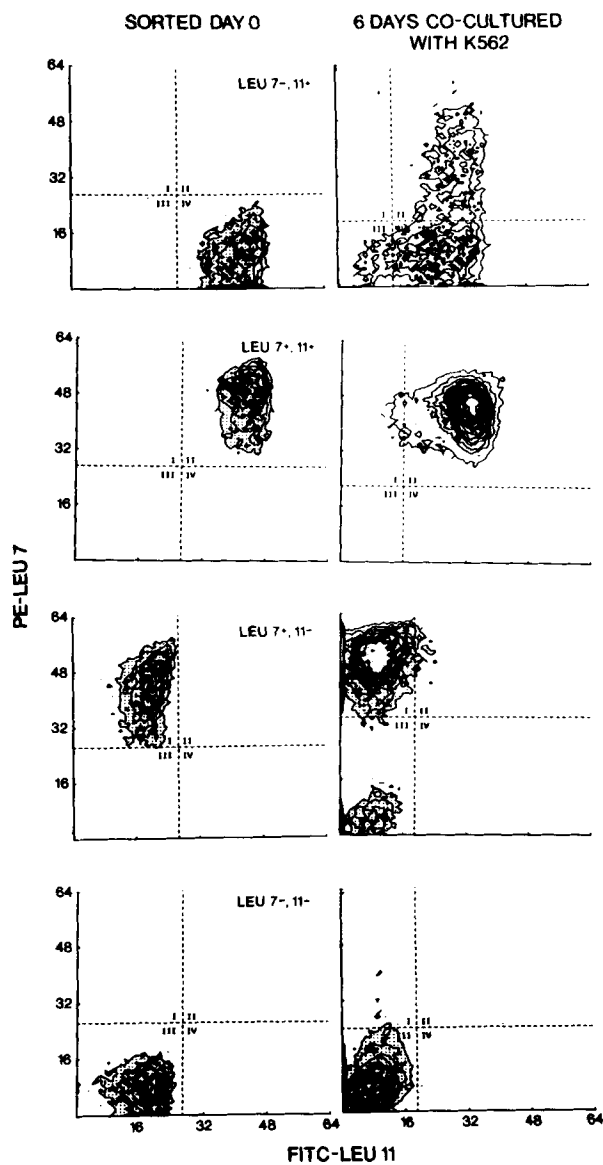


FIGURE 8. Antigenic profile of lymphocytes expressing the Leu-7 and/or Leu-11 antigens cocultured with K562. Lymphocyte subsets expressing the Leu-7 and/or Leu-11 antigens were isolated by two-color FACS cell sorting. Reanalysis of the purified populations are shown in the left panels (*sorted day 0*). All populations were >90% pure. These cells were cocultured with mitomycin C-treated K562. After 6 d, the viable cells were recovered and stained with FITC anti-Leu-11a and PE-avidin/biotin anti-Leu-7. Data are presented as described in Fig. 2. <5% of the cells in the Leu-7<sup>-</sup>11<sup>-</sup> population expressed the Leu-7 and/or Leu-11 antigen after coculture with K562. 95% of the Leu-7<sup>+</sup>11<sup>+</sup> cells coexpressed both antigens before and after stimulation with K562. In contrast, 37.7% of the Leu-7<sup>-</sup>11<sup>+</sup> cells demonstrated expression of Leu-7 after stimulation with K562. The small proportion of Leu-7<sup>-</sup>11<sup>-</sup> cells (14.9%) shown in the Leu-7<sup>+</sup>11<sup>-</sup> population after coculture with K562 was not observed in three subsequent experiments, and was likely explained by expansion or preferential survival of the 5% contamination of this population with Leu-7<sup>-</sup>11<sup>-</sup> cells during the sort.

from outgrowth of contaminating Leu-7<sup>+</sup>11<sup>+</sup> cells, since reanalysis immediately after the sort indicated <1% contamination with Leu-7<sup>+</sup>11<sup>+</sup> cells in the Leu-7<sup>-</sup>11<sup>+</sup> sorted fraction (Fig. 8). Additionally, the growth rate of the Leu-7<sup>-</sup>11<sup>+</sup> population was approximately twice that of the Leu-7<sup>+</sup>11<sup>+</sup> population (Fig. 6). The antigenic phenotype of the Leu-7<sup>+</sup>11<sup>+</sup> cells was unaffected by stimulation with K562; i.e., both Leu-7 and Leu-11 antigens were retained on essentially all cells (95%). Similarly, the Leu-7<sup>+</sup>11<sup>-</sup> cells neither gained expression of the Leu-11 antigen nor lost expression of Leu-7 as a consequence of coculture with K562 in three of four experiments (Fig. 8). Expression of the Leu-7 or Leu-11 antigens was not induced on cells in the Leu-7<sup>-</sup>11<sup>-</sup> population after K562 coculture (Fig. 8). Consistent results in the proliferation, cytotoxic, and antigenic phenotype assays were obtained in three experiments using blood from independent donors.

The lymphocyte subsets defined by Leu-7 and Leu-11 antigen expression, isolated to >95% purity by two-color FACS sorting, were stained with Giemsa and morphologically examined. The Leu-7<sup>-</sup>11<sup>+</sup> and Leu-7<sup>+</sup>11<sup>+</sup> subsets were homogeneous populations of typical LGL with prominent azurophilic granules (Fig. 9, A and C). After coculture with K562 for 6 d, the majority of cells in both subpopulations revealed primarily blast morphology, with abundant cytoplasm, numerous azurophilic granules, and large lobular euchromatic nuclei (Fig. 9, B and D). ~10–20% of the Leu-7<sup>-</sup>11<sup>+</sup> and Leu-7<sup>+</sup>11<sup>+</sup> cells cocultured with K562 displayed typical mitotic configurations (Fig. 9, B and D).

The Leu-7<sup>+</sup>11<sup>-</sup> cells, although also morphologically LGL, were generally smaller in size and consistently contained fewer azurophilic granules than either the Leu-7<sup>+</sup>11<sup>+</sup> or Leu-7<sup>-</sup>11<sup>+</sup> cells (Fig. 9E). Leu-7<sup>-</sup>11<sup>-</sup> cells were, morphologically, typical agranular small lymphocytes. After coculture with K562 for 6 d, the morphology of most Leu-7<sup>+</sup>11<sup>-</sup> and Leu-7<sup>-</sup>11<sup>-</sup> cells remained essentially unchanged (Fig. 9, F and H). However, a small percentage (5–10%) of cells with blast morphology were observed.

*Inhibition of Stimulation by mAb.* To determine the role of IL-2 receptors in the activation of granular lymphocytes by K562, anti-Tac (IL-2) receptor mAb or an isotype-matched nonreactive control IgG antibody were added to cocultures of mitomycin C-treated K562 and lymphocyte subpopulations, isolated by two-color cell sorting with FITC anti-Leu-11a and PE-avidin/biotin anti-Leu-7. Results from two independent donors are presented in Table II. The proliferative response of the Leu-7<sup>-</sup>11<sup>+</sup> against K562 was minimally inhibited (9%, Exp. 1; 6%, Exp. 2), whereas the response of the Leu-7<sup>+</sup>11<sup>+</sup> was partially inhibited (17%, Exp. 1; 18%, Exp. 2) by anti-Tac. In contrast, the mitogenic response of the T lymphocytes (Leu-7<sup>-</sup>11<sup>-</sup>) from both donors was completely inhibited (98%) by anti-Tac. Furthermore, the proliferation in the Leu-7<sup>-</sup>11<sup>-</sup> and Leu-7<sup>+</sup>11<sup>-</sup> cells against K562 in Exp. 2 also was completely inhibited by anti-Tac, suggesting that a different mechanism may be involved in the response of Leu-11<sup>-</sup> and Leu-11<sup>+</sup>

FIGURE 9. Morphology of lymphocytes expressing the Leu-7 and/or Leu-11 antigens after coculture with K562. Lymphocyte subsets expressing the Leu-7 and/or Leu-11 antigens were isolated to >95% purity by two-color FACS cell sorting. Immediately after sorting (A, C, E, and G) or after 6 d of coculture with mitomycin C-treated K562 (B, D, F, and H), Leu-7<sup>-</sup>11<sup>+</sup> (A, B), Leu-7<sup>+</sup>11<sup>+</sup> (C, D), Leu-7<sup>+</sup>11<sup>-</sup> (E, F), and Leu-7<sup>-</sup>11<sup>-</sup> (G, H) were stained with Giemsa and examined by light microscopy.  $\times 4,500$ .

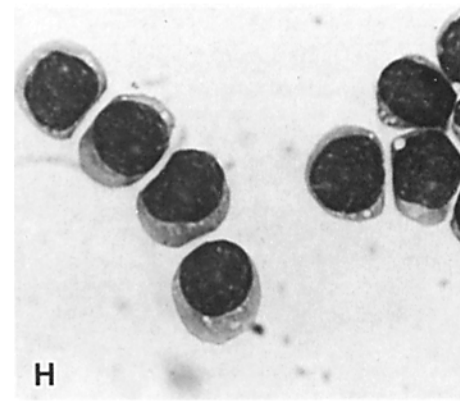
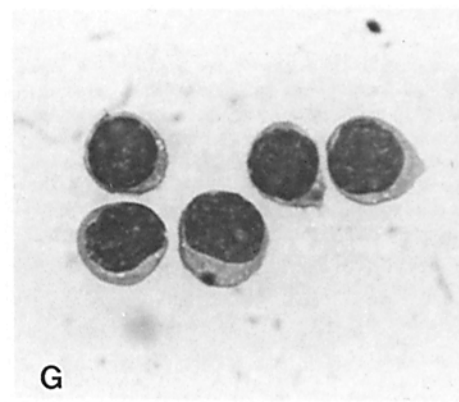
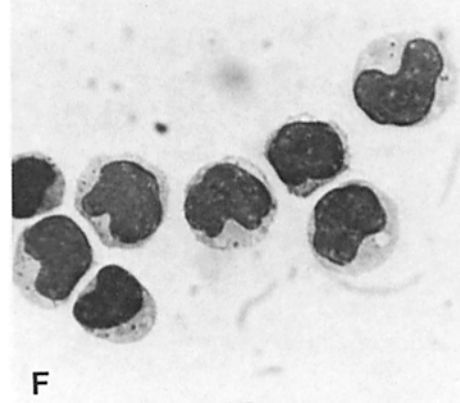
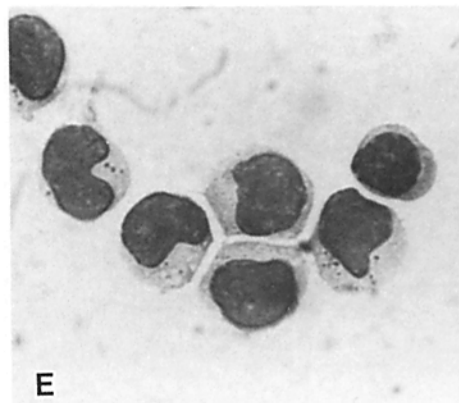
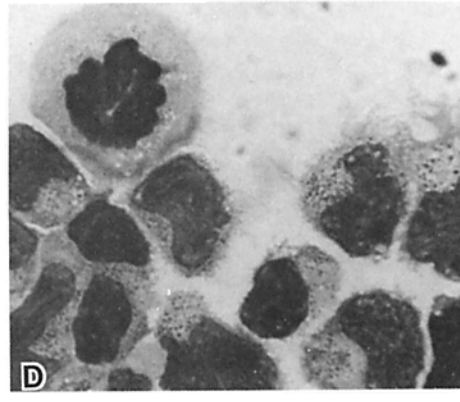
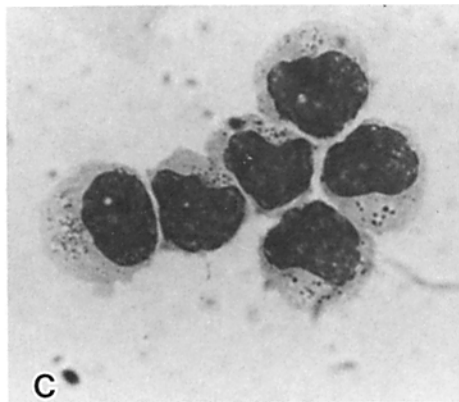
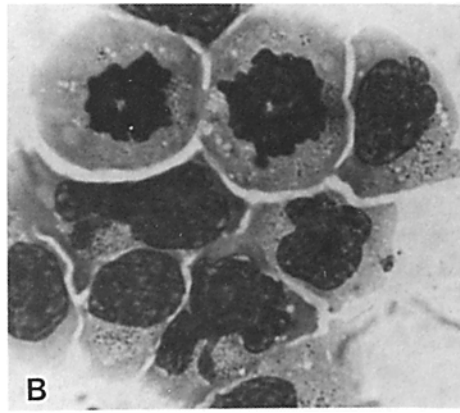
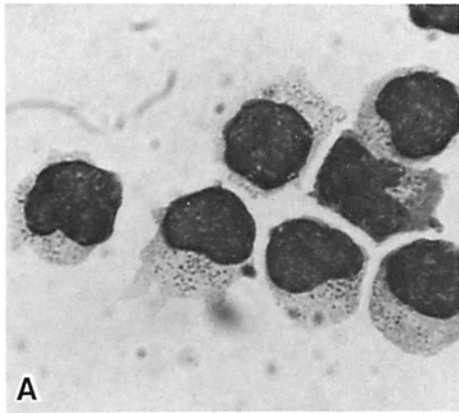


TABLE II  
Inhibition of Proliferation by mAb

Responder	Stimulator	Antibody	[ <sup>3</sup> H]thymidine <i>cpm</i>	Stimula- tion in- dex	Percent inhibition
Experiment 1					
Leu-7 <sup>-</sup> 11 <sup>+</sup>	None	None	200 ± 120	—	—
Leu-7 <sup>-</sup> 11 <sup>+</sup>	K562	Control IgG	9,300 ± 230*	46.5	—
Leu-7 <sup>-</sup> 11 <sup>+</sup>	K562	Anti-Tac	8,515 ± 115	42.6	9%
Leu-7 <sup>+</sup> 11 <sup>+</sup>	None	None	507 ± 213	—	—
Leu-7 <sup>+</sup> 11 <sup>+</sup>	K562	Control IgG	7,500 ± 120	14.8	—
Leu-7 <sup>+</sup> 11 <sup>+</sup>	K562	Anti-Tac	6,318 ± 198	12.5	17%
Leu-7 <sup>+</sup> 11 <sup>-</sup>	None	None	815 ± 180	—	—
Leu-7 <sup>+</sup> 11 <sup>-</sup>	K562	Control IgG	1,725 ± 271	2.1	—
Leu-7 <sup>+</sup> 11 <sup>-</sup>	K562	Anti-Tac	891 ± 176	1.1	92%
Leu-7 <sup>-</sup> 11 <sup>-</sup>	N	None	1,600 ± 223	—	—
Leu-7 <sup>-</sup> 11 <sup>-</sup>	K562	Control IgG	2,541 ± 211	1.6	—
Leu-7 <sup>-</sup> 11 <sup>-</sup>	K562	Anti-Tac	1,640 ± 142	1.0	96%
Leu-7 <sup>-</sup> 11 <sup>-</sup>	PHA	Control IgG	48,113 ± 416	30.1	—
Leu-7 <sup>-</sup> 11 <sup>-</sup>	PHA	Anti-Tac	1,988 ± 1,260	1.2	98%
Experiment 2					
Leu-7 <sup>-</sup> 11 <sup>+</sup>	None	None	420 ± 162	—	—
Leu-7 <sup>-</sup> 11 <sup>+</sup>	K562	Control IgG	12,810 ± 260	30.5	—
Leu-7 <sup>-</sup> 11 <sup>+</sup>	K562	Anti-Tac	11,989 ± 191	28.5	6%
Leu-7 <sup>+</sup> 11 <sup>+</sup>	None	None	342 ± 200	—	—
Leu-7 <sup>+</sup> 11 <sup>+</sup>	K562	Control IgG	6,580 ± 190	19.2	—
Leu-7 <sup>+</sup> 11 <sup>+</sup>	K562	Anti-Tac	5,420 ± 240	15.8	18%
Leu-7 <sup>+</sup> 11 <sup>-</sup>	None	None	500 ± 170	—	—
Leu-7 <sup>+</sup> 11 <sup>-</sup>	K562	Control IgG	3,260 ± 203	6.5	—
Leu-7 <sup>+</sup> 11 <sup>-</sup>	K562	Anti-Tac	620 ± 198	1.2	96%
Leu-7 <sup>-</sup> 11 <sup>-</sup>	None	None	921 ± 260	—	—
Leu-7 <sup>-</sup> 11 <sup>-</sup>	K562	Control IgG	6,824 ± 211	7.4	—
Leu-7 <sup>-</sup> 11 <sup>-</sup>	K562	Anti-Tac	1,231 ± 210	1.3	95%
Leu-7 <sup>-</sup> 11 <sup>-</sup>	PHA	Control IgG	18,420 ± 341	20	—
Leu-7 <sup>-</sup> 11 <sup>-</sup>	PHA	Anti-Tac	1,332 ± 240	1.4	98%

Leu-7 and Leu-11 subpopulations isolated by FACS sorting, as described in Fig. 6, were stimulated by coculture with K562 or PHA. Anti-Tac antibody and nonreactive IgG control antibodies were added to the cultures at a final concentration of 150 μg/ml. Cultures were pulsed overnight with 1 μCi [<sup>3</sup>H]thymidine and were harvested on day 6. The stimulation index is defined in Table 1.

\* Background thymidine incorporation by the mitomycin C-treated K562 or CCRF-SB tumor cells (tumor inactivation control) was subtracted from the raw counts to determine the specific incorporation by the effector cell population. Percent inhibition is defined as: 100 × [(cpm with K562 and control IgG - cpm without stimulator and antibody) - (cpm with K562 and anti-Tac - cpm without stimulator or antibody)] / (cpm with K562 and control IgG - cpm without stimulator or antibody).

cells against K562. As described previously (14), anti-Tac antibody did not inhibit the cytotoxic activity of NK cells.

### Discussion

Leu-11<sup>+</sup> lymphoblasts (6) were a minor population in MLTR cultures using an allogeneic B cell line as the stimulator. In general, allogeneic B cell lines are relatively insensitive to NK-mediated cytotoxicity. We questioned whether Leu-11<sup>+</sup> cells could be preferentially stimulated if an NK-sensitive tumor cell line was used as the stimulator in an MLTR culture. Poros and Klein (33) have reported that coculture of peripheral blood mononuclear cells with an NK-sensitive tumor cell, K562, results in the preferential activation of lymphoblasts that express Fc receptors and are highly cytotoxic for K562. In contrast (33), stimulation with allogeneic peripheral blood lymphocytes preferentially stimulates typical E rosette-positive lymphoblasts that lack Fc receptor expression. We have confirmed these observations (34). Coculture of mitomycin C-treated K562 with either unseparated peripheral blood lymphocytes or Percoll gradient-enriched low density lymphocytes results in proliferation and activation of cells expressing the Leu-11 antigen, but usually lacking pan T cell antigens (34). Depletion studies (34) with antibody and complement show that the peripheral blood precursor of the activated Leu-11<sup>+</sup> cell expresses the Leu-11 antigen. The structure on K562 responsible for activation of NK cells is unknown, but it is important to note that K562 lacks expression of both class I and II MHC antigens on the cell surface. We do not know if K562 cells stimulate NK cells through release of a soluble product, but are exploring this possibility.

An important unresolved issue was whether or not NK cells could be stimulated directly by coculturing purified Leu-11<sup>+</sup> cells with K562. We have demonstrated that it is possible to selectively stimulate Leu-11<sup>+</sup> and Leu-11<sup>-</sup> responders with K562 and CCRF-SB, respectively. Stimulation of the highly purified Leu-11<sup>+</sup> cells with K562 resulted in blastogenesis, enhanced cytotoxicity against NK-sensitive and -insensitive targets, and expression of cell surface activation antigens (class II MHC antigens and transferrin receptor) on a portion of the cells. The observation that Leu-11<sup>+</sup> granular lymphocytes were preferentially stimulated by K562 implies that NK cells can demonstrate specificity in the recognition process. These experiments also suggest that Leu-11<sup>+</sup> cells may be capable of stimulation and proliferation in the absence of T lymphocytes, exogenous growth factors, or accessory cells. However, the possibility that a small number of contaminating monocytes (<1%) or T lymphocytes (<5%) influenced the response of the Leu-11<sup>+</sup> lymphocytes against K562 cannot be excluded. We are currently investigating the influence of adding monocytes and T cells to the cultures, and determining the range of tumor cell lines that are capable of initiating activation.

The relationship between NK cells and T lymphocytes has been a controversial issue. The arguments range from the view that cytotoxic T cells "degenerate" into NK cells, to the idea that NK cells are primitive T lymphocytes that do not require thymic processing. We have been unable to induced expression of Leu-11 antigen by coculturing purified T (Leu-11<sup>-</sup>) lymphocytes with K562. Although, typically, there was little cytotoxic or proliferative activity in the Leu-

$11^-$  population after stimulation with K562, we observed significant levels of proliferation and cytotoxicity in some individuals. This is consistent with reports that some IL-2-dependent clones that express T cell antigen receptor and CD3 antigen, but lack Fc receptors (Leu-11), can lyse NK-sensitive tumors (22–25). By contrast, essentially all Leu-11<sup>+</sup> cells stimulated by coculture with K562 retained expression of Leu-11 antigen, were highly cytotoxic for NK-sensitive and -insensitive tumor cells, and usually did not acquire expression of the pan T cell antigens, CD3. The relationship between the K562-reactive CD3<sup>+</sup> lymphocytes and Leu-11<sup>+</sup> lymphocytes is unknown.

Within the LGL population there is considerable heterogeneity in expression of cell surface antigens (2, 3, 28, 32, 34, 35). Previously, we (2) and other investigators (36) have demonstrated that four distinct subsets of lymphocytes can be defined by expression of the Leu-7 and/or Leu-11 antigens. These subsets were purified and cocultured with K562. One interpretation consistent with the data is that the Leu-7 antigen appears late in the differentiation of NK cells. This relationship is depicted in Fig. 10. In this model, the Leu-7<sup>-</sup>11<sup>+</sup> cells, when activated with an appropriate signal such as stimulation with K562, rapidly proliferate, regenerating more cells with the Leu-7<sup>-</sup>11<sup>+</sup> phenotype and some cells that acquire the Leu-7 antigen. Our experiments unequivocally demonstrate that Leu-7<sup>+</sup>11<sup>+</sup> lymphocytes can arise from Leu-7<sup>-</sup>11<sup>+</sup> precursors, and that there is a developmental relationship between these two phenotypically distinct subsets. Based on the observation that Leu-7<sup>+</sup>11<sup>+</sup> cells derived from peripheral blood consistently demonstrate lower proliferative capacity than the Leu-7<sup>-</sup>11<sup>+</sup> cells, we conclude that these cells may represent an NK cell that is more mature and has less regenerative capacity than the Leu-7<sup>-</sup>11<sup>+</sup> cell.

Abo and colleagues (36, 37) have proposed that the Leu-7 antigen is an early marker of NK cell differentiation and that the Leu-7<sup>+</sup>11<sup>-</sup> population may represent a “pre-NK” cell. They have predicted that the Leu-7<sup>+</sup>11<sup>-</sup> cells acquire the Leu-11 antigen during maturation and that the Leu-7 antigen may be lost upon activation of the Leu-7<sup>+</sup>11<sup>+</sup> subset (36). This interpretation is inconsistent with results from allogeneic MLTR experiments (6) and our present study. We have been unable to induce significant NK cell-mediated cytotoxic function or

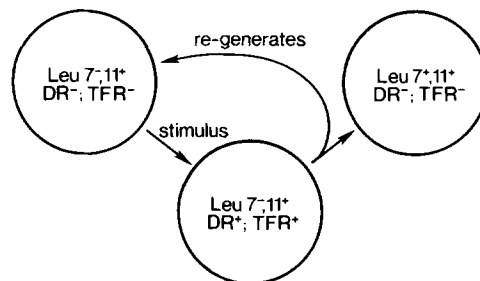


FIGURE 10. A model for the differentiation of Leu-11<sup>+</sup> lymphocytes. In this model, Leu-11<sup>+</sup>7<sup>-</sup> lymphocytes can be activated by an appropriate stimulus, such as interaction with K562 cells. As a consequence, a proportion of the cells acquire activation antigens (e.g., HLA-DR and transferrin receptor, TFR). During the activation process, Leu-7<sup>-</sup>11<sup>+</sup> proliferate, regenerating more cells with this antigenic phenotype. Additionally, Leu-7 antigen is induced on a proportion of the lymphocytes (20–40%).



expression of the Leu-11 antigen on the Leu-7<sup>+</sup>11<sup>-</sup> cells by stimulation with K562 or recombinant IL-2 (14). Thus, the relationship of the Leu-7<sup>+</sup>11<sup>-</sup> granular lymphocytes to Leu-11<sup>+</sup> cytotoxic NK cells is unclear. Another substantial difference between Leu-7<sup>+</sup>11<sup>-</sup> and Leu-11<sup>+</sup> cells is that the former all express the CD3 antigen (2), a glycoprotein associated with the T cell antigen receptor complex (38, 39). At present, there is no direct experimental evidence that these Leu-7<sup>+</sup>, CD3<sup>+</sup> lymphocytes are developmentally related to the Leu-11<sup>+</sup> lymphocyte population.

In this study, we also investigated whether anti-Tac (IL-2 receptor) mAb influenced the activation and proliferation of Leu-11<sup>+</sup> cells in response to K562. Of particular interest, anti-Tac only minimally inhibited the proliferation of Leu-7<sup>-</sup>11<sup>+</sup> cells against K562 and only partially inhibited the response of Leu-7<sup>+</sup>11<sup>+</sup> cells. Furthermore, anti-Tac mAb did not react with Leu-11<sup>+</sup> lymphoblasts stimulated with K562. In contrast, the PHA-induced mitogenic response of T (Leu-11<sup>-</sup>) cells from the same individual and the proliferative response of the Leu-11<sup>-</sup> cells against K562 was completely inhibited by anti-Tac antibody. These data imply either that IL-2 and the IL-2 receptor are not involved in Leu-11<sup>+</sup> NK cell stimulation with K562 or that NK cells possess a distinct IL-2 receptor that cannot be regulated by antibodies against the Tac epitope. The latter possibility is supported by reports that anti-Tac antibody failed to inhibit the augmentation of NK activity by recombinant IL-2 (14, 20). We are investigating whether IL-2 is involved in the stimulation of Leu-11<sup>+</sup> cells with K562.

The observation that Leu-11<sup>+</sup> lymphocytes can respond directly to an NK-sensitive tumor cell makes this assay an excellent model with which to study the function of NK cells. It should thus be possible to explore the heterogeneity in target cell recognition, surface structures involved in the activation process, and the pathway of differentiation.

### Summary

A subpopulation of low density granular lymphocytes that express the natural killer (NK) cell-associated Leu-11 antigen (IgG Fc receptor) were stimulated directly by coculture with an NK-sensitive tumor cell, K562. T lymphocytes (Leu-11<sup>-</sup>) responded only weakly when cocultured with K562. The response of Leu-11<sup>+</sup> cells apparently did not require exogenous factors or accessory cells. The K562-activated cells retained expression of Leu-11 antigen, acquired activation antigens, and were highly cytotoxic against NK-sensitive and -insensitive tumor cells. Anti-IL-2 receptor monoclonal antibody minimally inhibited the activation of Leu-11<sup>+</sup> cells by K562, but completely inhibited the phytohemagglutinin-induced activation of the Leu-11<sup>-</sup> cells from the same individual. Leu-11<sup>+</sup> cells can be divided into Leu-7<sup>-</sup>11<sup>+</sup> and Leu-7<sup>+</sup>11<sup>+</sup> subpopulations using anti-Leu-7 antibody. These subsets were separated by two-color fluorescence-activated cell sorting and cocultured with K562. Proliferation by Leu-7<sup>-</sup>11<sup>+</sup> cells was significantly greater than by Leu-11<sup>+</sup>7<sup>+</sup> cells. Leu-7<sup>+</sup>11<sup>-</sup> granular lymphocytes and T lymphocytes (Leu-7<sup>-</sup>11<sup>-</sup>) typically proliferated only weakly when cocultured with K562. A proportion of the Leu-7<sup>-</sup>11<sup>+</sup> cells acquired Leu-7 antigen after stimulation with K562, whereas the phenotype of Leu-7<sup>+</sup>11<sup>+</sup>, Leu-7<sup>+</sup>11<sup>-</sup>, and Leu-7<sup>-</sup>11<sup>-</sup> subsets was unaffected. These results demonstrate a

developmental relationship between the Leu-7<sup>-</sup>11<sup>+</sup> and Leu-7<sup>+</sup>11<sup>+</sup> lymphocytes and suggest that Leu-7 antigen may be expressed late in the differentiation pathway of NK cells. The direct activation of highly purified Leu-11<sup>+</sup> cells by coculture with K562 provides an in vitro model with which to study the activation and maturation of human NK cells.

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