Induction of Nonspecific Cell-Mediated Cytotoxicity: A Multisignal Event and its Cellular Regulation*

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A. Introduction

Nonspecific cytotoxic cells provide a major line of defense against tumor. While natural killer (NK) cells display spontaneous, non-MHC-restricted killing activity, both NK and non-NK lymphocytes can be induced by lymphokines to exhibit enhanced nonspecific cytotoxicity against tumor, including NK-resistant targets [1–3]. The latter activity is mediated by a heterogeneous cell population commonly termed lymphokine-activated killer (LAK) cells [2–4]. According to the initial concept, nonspecific LAK killing is generated via exposure to interleukin 2 (IL-2) of peripheral blood lymphocytes [5, 6]. Most of the LAK activity appears to be mediated by NK cells stimulated with IL-2; however, recent studies suggest that induction of MHC-unrestricted lymphokine-activated killing is a more complex phenomenon requiring a multitude of cellular and noncellular signals [2–4, 7–10].

In our study, we approached the LAK phenomenon by asking the following questions: (a) what are the cell types mediating LAK precursor and/or effector function? (b) What are the signals needed for the induction of nonspecific killing? Do different LAK precursor populations require different induction signals? (c) What is the regulatory role of various mononuclear cell subsets in the induction of nonspecific cytotoxicity? In order to answer these questions, we isolated and depleted NK cells, T lymphocytes, and monocytes, respectively, from fresh human peripheral blood. By culturing these cell populations under various conditions, we could define the role of both cellular and noncellular signals for the induction of MHC-unrestricted killing in human peripheral blood.

B. Methods

Production and characterization of monoclonal antibodies (mAb) used in this study have been described elsewhere [11–18]. NK cells, T-lymphocytes, and monocytes were selectively removed and purified from peripheral blood mononuclear cells (PBMC) using techniques that have been detailed previously [19–21]. In brief, for selective removal of NK cells and T cells, PBMC were preincubated with mAbs N901, and OKT3 plus H65, respectively. Reactive cells were then depleted by immunoadherence to plastic plates previously coated with affinity-purified goat anti-mouse IgG. NK-depleted fractions were additionally treated with the lysomotropic agent L-leucinemethylester (LeuOMe) [22]. Monocytes were removed from fresh PBMC by adherence to plastic petri dishes. After col-

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lecting the nonadherent cells, adherent fractions were harvested by incubation with medium plus EDTA. Fractions of monocyte-depleted PBMC were purified for T-lymphocytes and for NK cells. For isolation of T cells, the indirect immunoadherence technique was employed using mAbs B1, B4, MY4, MY8, MO-1, and N901; nonadherent cells were subsequently treated with LeuOMe to ensure complete removal of all NK cells and monocytes. For separation of NK cells from peripheral blood, PBMC were incubated with mAbs OKT3, H65, B1, B4, MY4, and MY8; positive cells were depleted by immune rosetting with bovine red blood cells that were coated with affinity-purified goat anti-mouse IgG by $CrCl_3$ linkage [23]. For immunofluorescence analyses [24], cells were incubated separately with mAbs and purified mouse IgG controls, then stained with fluorescein isothiocyanate conjugated $F(ab)_2$ fragments of goat anti-mouse IgG and analyzed on a FACScan flow cytometer. Phenotypic analyses of isolated cell fractions revealed that all purified and depleted populations contained under 2% (i.e., no detectable) inappropriate cells. To measure nonspecific NK and LAK cytotoxicity, a standard 4-h ^{51}Cr-release assay [25] was performed using two MHC class I negative tumor cell lines (K562, Daudi) [26, 27] as targets.

C. Results

I. NK Cells as Nonspecific Precursor and Effector Cells

As shown in Fig. 1, fresh thoroughly purified NK cells lysed approximately 50% of the NK-sensitive K562 cell line, as compared to 0% lysis of NK-resistant, LAK-sensitive Daudi targets (50:1 E:T ratio). Following 5-day stimulation with recombinant IL-2 (1000 U/ml), cytotoxicity mediated by isolated NK cells was measured at 100% against Daudi and 16% against K562 (50:1 E:T ratio). When compared to unfractionated mononuclear cells, fresh unstimulated NK cells exhibited an approximately tenfold higher non-specific killing activity, while day 5 IL-2 induced cytotoxicity was measured at equal levels in both purified NK and unseparated PBMC cultures. Thus, stringent isolation of fresh NK cells did not result in a detectable enrichment for nonspecific lymphokine-activated killing as defined by day 5 Daudi cell lysis. However, rigorous depletion of NK cells from PBMC prior to IL-2 induction completely abrogated spontaneous as well as IL-2 activated non-MHC-restricted cytotoxicity (Fig. 2).

II. T-Lymphocytes as LAK Precursor and Effector Cells

Figure 3A gives the phenotypic analyses of highly purified T-lymphocytes that were initially depleted of NK cells and grown in short-term culture in the presence of IL-2 or IL-2 plus mitogen (PHA-M). While IL-2 by itself was unable to induce the generation of nonspecific cytotoxic cells, costimulation of IL-2 induced CD3 lymphocytes via the T3/Ti receptor antigen resulted in the generation of T-lymphocytes that expressed NK-related surface molecules (N901, H25) [17, 18] and concomitantly acquired both NK and LAK cytotoxicity (Fig. 3). As shown in Fig. 2B, when using unseparated peripheral blood mononuclear cells, a stringent removal of T cells on day 0 resulted in a partial, though significant, depletion of IL-2 induced nonspecific cytolysis of Daudi. This suggested that a proportion of N901-negative T-lymphocytes represents a precursor population to MHC-unrestricted lymphokine-activated killer cells. While mediating LAK precursor functions, $CD3^+$ T cells were also demonstrated to account for approximately 30%–50% of IL-2 induced MHC-unrestricted cytotoxicity generated in unfractionated cultures comprising NK cells, T-lymphocytes, and monocytes; evidence supporting this was provided in cell depletion experiments whereby NK cells

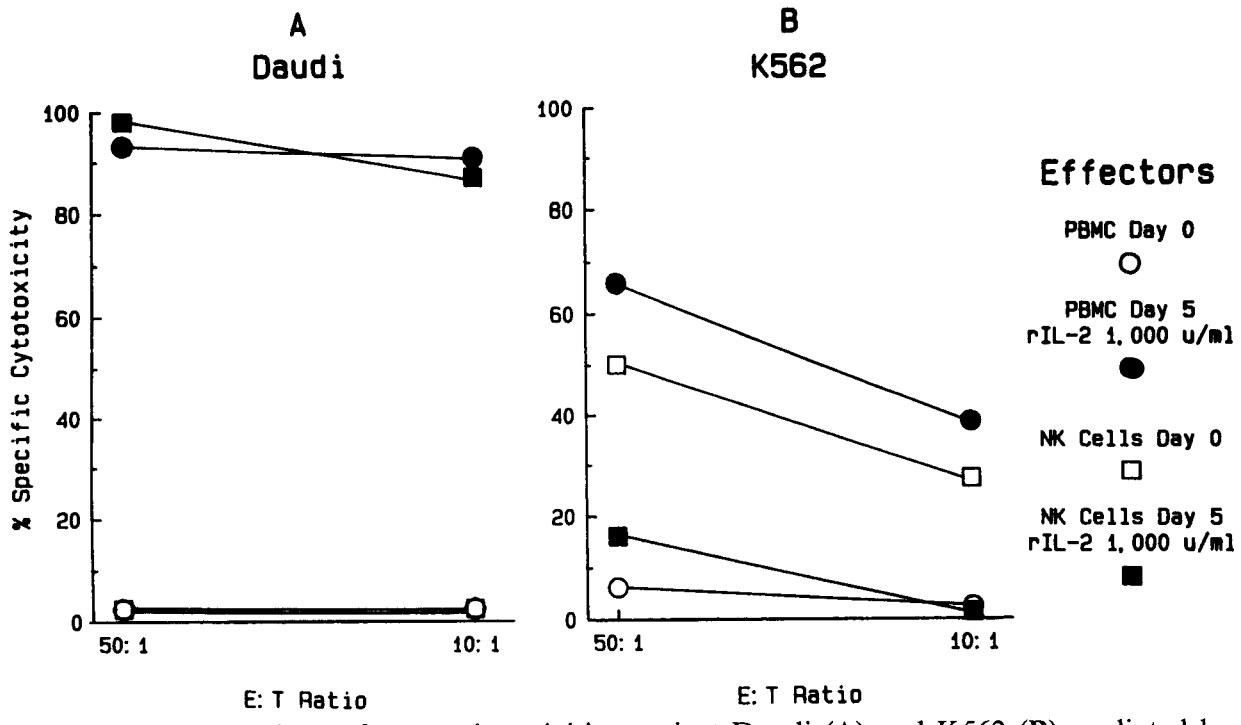

Fig. 1 A, B. Comparison of cytotoxic activities against Daudi (**A**) and K562 (**B**) mediated by unseparated PBMC before and after 5-day IL-2 activation, or by purified NK cells before and after 5-day stimulation with IL-2

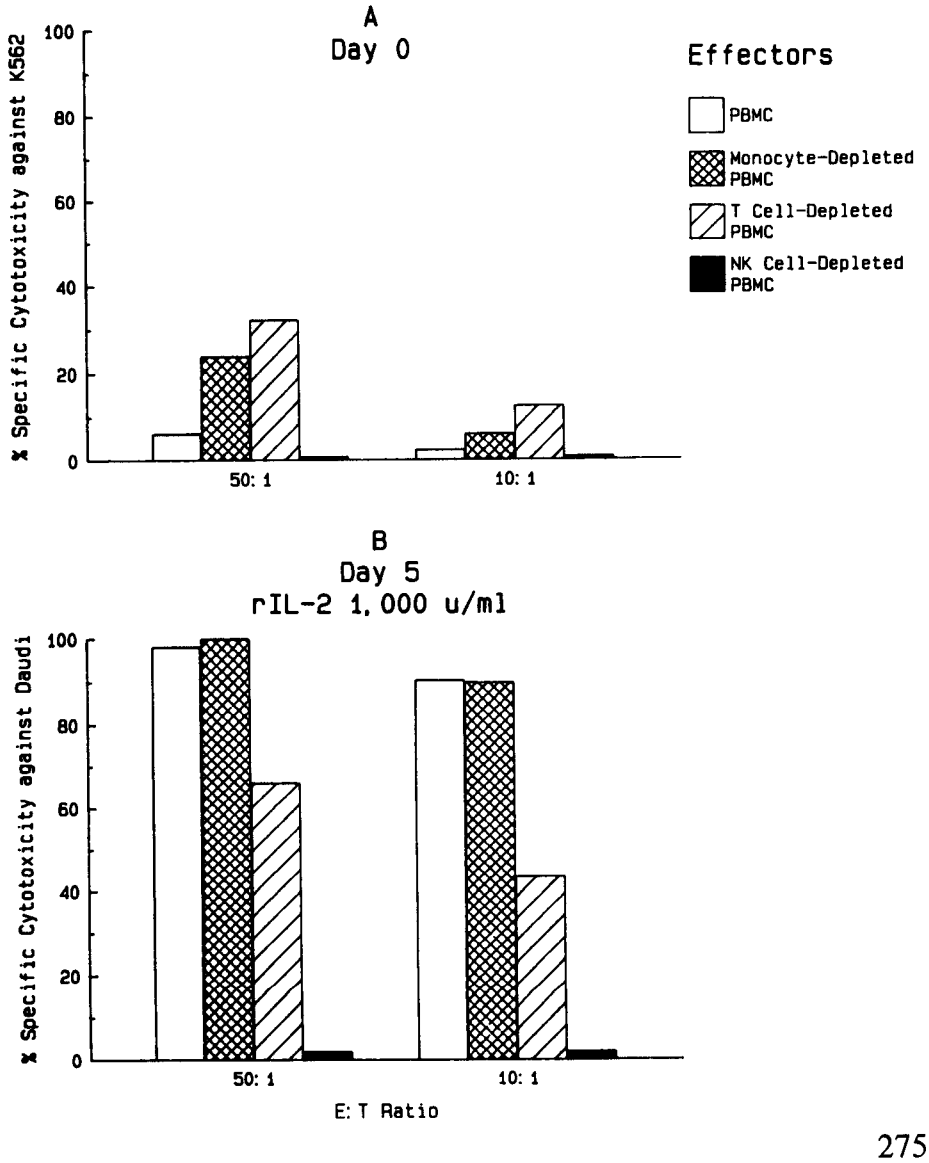

Fig. 2 A, B. Cytotoxicity before (**A**) and after (**B**) 5-day IL-2 incubation mediated by unseparated PBMC and PBMC that were depleted on day 0 of NK cells, T cells, or monocytes

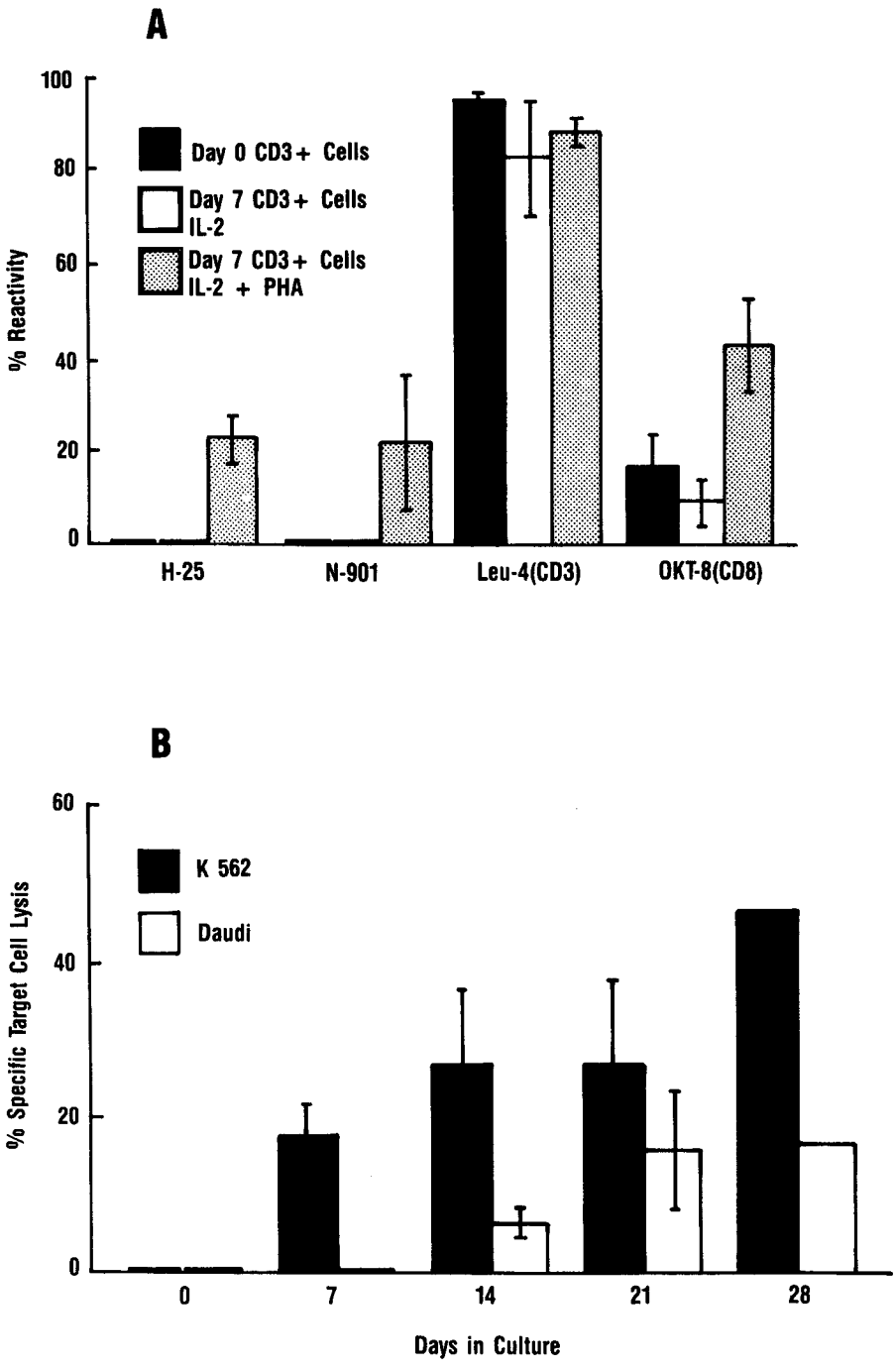

Fig. 3A, B. Phenotypic (**A**) and functional (**B**) analyses of purified $CD3^+$ T-lymphocytes following induction with IL-2 in the absence (**A**) or presence (**A, B**) of PHA mitogen

and monocytes were stringently removed from day 5 IL-2 induced cultures (Fig. 4).

III. Monocytes as Regulators of Nonspecific Cytotoxicity

PBMC, initially depleted of all detectable monocytes, showed an increased relative cytotoxicity against K562 while lymphokine-activated killing of Daudi was essentially unchanged (Fig. 2). In short-term culture with IL-2, monocytes did not exhibit any nonspecific cytotoxicity against tumor cells. Nonetheless, proliferation of MHC-unrestricted killer cells was augmented significantly when purified NK cells were cultured with IL-2 in the presence of autologous monocytes (Table 1). Consistently, the cumulative nonspecific cytolytic activity induced in NK cultures in response to IL-2 was increased approximately twofold with autologous monocytes present (Fig. 5).

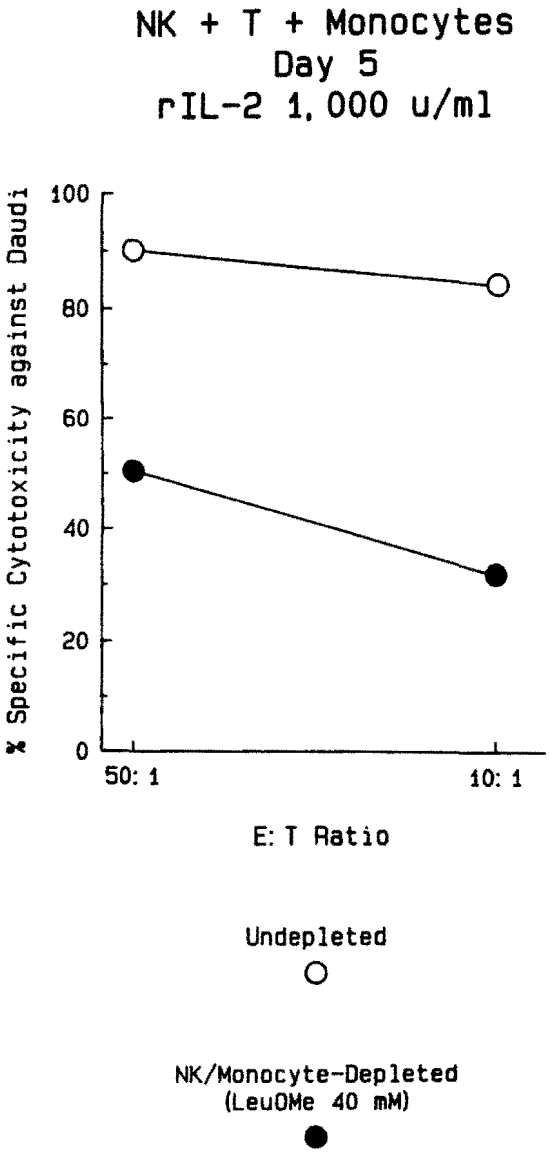

Fig. 4. IL-2 induced non-MHC-restricted cytotoxicity mediated by day 5 undepleted, and NK cell plus monocyte depleted effector populations that were generated in purified NK cultures in the presence of equal numbers of autologous T cells and monocytes

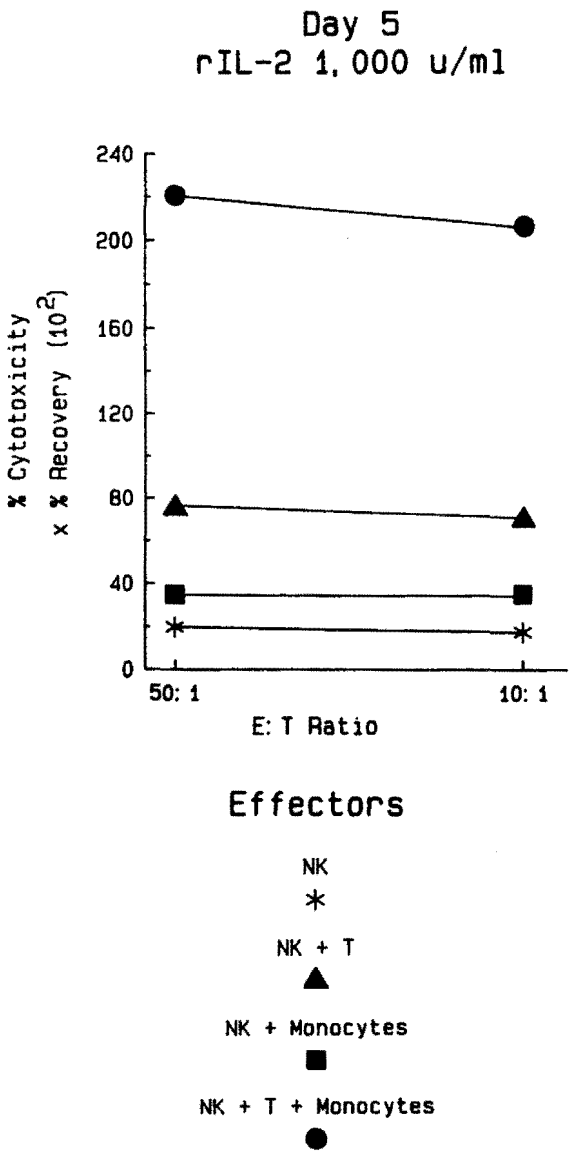

Fig. 5. Cumulative lymphokine-activated cytotoxicity (percent cytolysis × percent recovery) generated in IL-2 cultures of highly purified NK cells that were incubated alone, or in the presence of T cells, monocytes, or T cells plus monocytes

IV. T Cells and Monocytes as Coregulators of MHC-Unrestricted Lymphokine-Activated Killing

When added to NK cells on day 0, autologous T-lymphocytes could induce an approximately fourfold increase in the absolute number of nonspecific effector cells generated upon induction with IL-2 (Table 1). While percent cytotoxicity against Daudi was essentially unchanged (data not shown), addition of T-lymphocytes to freshly isolated NK cells resulted in a significant (i.e., more than threefold) enhancement of cumulative non-MHC-restricted killing (percent cytotoxicity × percent recovery) following 5-day IL-2 activation (Fig. 5). As shown in Table 1, costimulation of NK cells, T cells, and monocytes with IL-2 had an even more pronounced effect on the expansion of cytolytic effectors in response to cytokine. Compared to highly purified NK cells alone, approximately 15- and

Table 1. Proliferation and cell recovery after 5-day incubation with rIL-2 of highly purified subpopulations isolated from human peripheral blood

Peripheral blood subpopulation	[^{3}H]-Thymidine incorporation[a] (cpm; mean ± 1 SD)	Cell recovery[b] (%)
NK cells	914 ± 301	20
T cells	211 ± 107	68
Monocytes	565 ± 162	10
NK cells plus T cells[c]	1482 ± 316	80
NK cells plus monocytes	3162 ± 802	35
T cells plus monocytes	3466 ± 860	60
NK cells plus T cells plus monocytes	14041 ± 2718	245

[a] 10^4 cells each were pulsed with [^{3}H]-thymidine for 4 h.
[b] Cell recovery as compared to day 0 was assessed by trypan blue dye exclusion test.
[c] After purification, the various subpopulations were admixed at equal ratios prior to rIL-2 culture.

12-fold higher levels of proliferation and cell recovery, respectively, were measured in NK cultures grown in the presence of equal numbers of T cells plus monocytes (Table 1). Thus, autologous T-lymphocytes and monocytes, when added to fresh NK cells, were able to induce a dramatic (i.e., more than tenfold) increase in total nonspecific cytolytic activity generated upon 5-day stimulation with IL-2.

D. Discussion

Both NK and LAK cells are functionally defined by their non-MHC-restricted cytotoxicity against tumor [1–3]. In the present study, the main findings about the induction of nonspecific cell-mediated cytotoxicity in human peripheral blood are the following: (a) NK cells ($N901^+$ $CD3^-$) and T-lymphocytes ($CD3^+$ $N901^-$) can function separately as precursor cells to MHC-unrestricted LAK cells; (b) non-NK ($N901^-$) T cells can give rise to a subset of NK-like ($N901^+$) T-lymphocytes that coexpress NK (N901) and T-cell (CD3) specific antigens and concomitantly acquire NK and LAK cytotoxicity in response to IL-2; (c) while in NK cells, IL-2 provides a sufficient signal for the induction of nonspecific lymphokine-activated cytotoxicity, additional activation via the T3/Ti receptor antigen is needed in order to induce non-MHC-restricted LAK activity in a proportion of mature $N901^-$ T-lymphocytes; (d) T cells do not only mediate but also regulate LAK precursor and effector functions whereby cumulative nonspecific cytolysis (percent cytotoxicity × recovery) is increased more than threefold in IL-2 induced NK cultures grown in the presence of $CD3^+$ cells; (e) monocytes, when coincubated with NK cells, augment the cumulative LAK activity generated in NK cultures upon stimulation with IL-2; and (f) monocytes and T cells strongly synergize in enhancing the generation and activation of NK-derived MHC-unrestricted killer cells, thus producing a dramatic (i.e., more than tenfold) increase in the total nonspecific killing induced by IL-2.

While the ontogeny of IL-2 activated killer cells continues to be controversial [2, 3, 8–10], there is pertinent evidence that the phenomenon of LAK cells defines a cellular function rather than a distinct cell type [2–4, 6]. In this study, we show that a variety of different mononuclear cell subsets contribute to the generation and activation of non-MHC-re-

stricted IL-2 induced killer cells. Based on the observation of various cellular and noncellular signals for the induction in human peripheral blood of nonspecific cell-mediated cytotoxicity, we propose that activation of unrestricted cytolytic cells constitutes a multisignal event tightly regulated at the cellular level. Our data indicate that application of IL-2 has both direct and indirect effects, whereby NK cells, T-lymphocytes, and monocytes may be induced to synergize in generating nonspecific killing activity in a subset of cells presenting with NK or NK/T cell phenotype. As to the ontogeny of NK and LAK cells, the present results also suggest that a proportion of NK-like peripheral blood mononuclear cells can be derived from mature T-lymphocytes.

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