Brief Definitive Report

ACTIVATION OF NATURAL KILLER CELLS VIA THE p75 INTERLEUKIN 2 RECEPTOR

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IL-2 is a 15,000-dalton glycoprotein that is secreted by T lymphocytes. IL-2 binds to a membrane receptor on lymphocytes and monocytes and initiates activation, proliferation, and differentiation by a process that has not been completely delineated (1). The IL-2R is composed of at least two subunits, the p55 (CD25/Tac) and p75 glycoproteins (2–4). The p55 subunit binds IL-2 with low affinity ($K_d \sim 10^{-8}$) and the p75 subunit binds with intermediate affinity ($K_d \sim 10^{-9}$), whereas a dimeric receptor composed of both p55 and P75 binds IL-2 with high affinity ($K_d \sim 10^{-12}$) (2–4). Cells may express p55 alone, p75 alone, or both subunits (2–4). p75 IL-2R is responsible for signal transduction, whereas p55 primarily functions to create a high affinity receptor by association with p75 (5, 6).

Human peripheral blood NK cells ($CD3^-CD16^+$ lymphocytes) are rapidly activated by IL-2, resulting in augmented cytotoxic activity, induction of activation antigens, and proliferation (7–9). Resting NK cells do not express p55 IL-2R (CD25), suggesting that the response is mediated by another IL-2 binding protein (7, 8). Biochemical crosslinking experiments using ^{125}I-labeled IL-2 have demonstrated the presence of 70/75-kD proteins on the membrane of NK cells (10, 11). Recently, mAbs have been produced against the p75 IL-2R (12, 13). Thus, it is now possible to directly assess the expression and function of this structure in IL-2-mediated activation. In the present study, we have examined the role of the P75 and p55 IL-2R in NK cell activation.

Materials and Methods

Cell Culture. PBMC were isolated using Ficoll/Hypaque. After plastic adherence and passage through nylon wool to remove monocytes and B cells, respectively, lymphocytes were fractionated by centrifugation on discontinuous gradients consisting of 30 and 40% Percoll (Pharmacia Fine Chemicals, Piscataway, NJ), as described (14). Low buoyant density cells isolated from the interface of Percoll gradients consisted of ~40–50% NK cells ($CD3^-$, $CD16^+$) and 50–60% T cells ($CD3^+$, $CD16^-$). Cells were cultured in RPMI 1640 (M. A. Bioproducts, Walkersville, MD), 4% heat-inactivated horse serum (KC Biologicals, Lexena, KS), 1 mM L-glutamine (Gibco Laboratories, Chagrin Falls, OH), and antibiotics. rIL-2 was generously provided by Cetus Corp., Emeryville, CA. For proliferation assays, cells were cultured in 96-well microtiter plates and were harvested on day 4, after overnight labeling with [^{3}H]thymidine (1 μCi/well) (Amersham Corp., Arlington Heights, IL).

Monoclonal Antibodies. TU27 mAb reacts with the p75 subunit of the IL-2R and inhibits the binding of IL-2 to the receptor (12). Anti-IL-2R (CD25) (clone 2A3) reacts with the p55 subunit of the IL-2R. Leu series mAbs were produced by Becton Dickinson Monoclonal

Center, Inc. Phycoerythrin (PE)-conjugated rat anti-mouse IgG1 mAb and anti-Leu 23 mAb were generously provided by Mr. David Buck and Dr. Anne Jackson (Becton Dickinson), respectively.

Immunofluorescence and Flow Cytometry. Immunofluorescence and flow cytometry were performed as described (15). Samples were analyzed using a FACScan and cell sorting was performed using a FACS IV (Becton Dickinson Immunocytometry Systems, San Jose, CA).

Cytotoxicity. Cytotoxicity was measured by a 4-h ^{51}Cr-release assay using the NK-resistant Colo-205 colon carcinoma cell line as target (14).

Results and Discussion

Preferential Expression of p75 IL-2R on NK Cells. Peripheral blood leukocytes were stained with anti-p75 IL-2R mAb to determine distribution of this receptor on normal, resting cells. p75 IL-2R was detected on a small proportion of peripheral blood lymphocytes (5–15% of lymphocytes, depending on the donor), but was not detected on monocytes or granulocytes (Fig. 1 *A*). Within the lymphocyte population, p75 IL-2R was expressed preferentially on CD16$^+$ NK cells (Fig. 1 *B*). Examination of the contour plot correlating CD16 and p75 IL-2R indicated that essentially all CD16$^+$ NK cells expressed low levels of this antigen, whereas few (<5%) of CD16$^-$ lymphocytes, including B and T lymphocytes, demonstrated detectable levels of p75 IL-2R. In most donors, CD16 is expressed exclusively on CD3$^-$ NK cells, and is not present on T or B lymphocytes (15). Consistent with prior reports (7, 8), p55 IL-2R was not detected on CD16$^+$ NK cells (not shown). Expression of p75 IL-2R on NK cells, but not a substantial proportion of B or T lymphocytes, was observed in more than 10 normal donors.

Affect of Anti-p75 IL-2R mAb on NK Cell Activation, Cytotoxicity, and Proliferation. The finding that NK cells constitutively express p75 IL-2R is consistent with prior observations that IL-2 alone preferentially activates resting, peripheral blood NK cells (7, 8, 10), but not a substantial proportion of T or B lymphocytes. Stimulation of

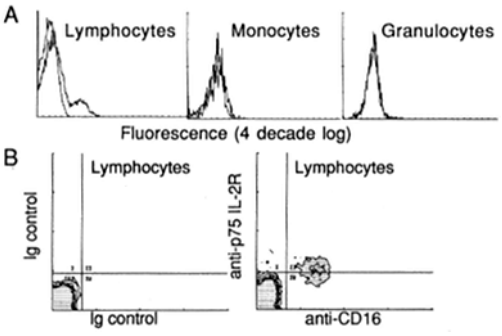

FIGURE 1. Expression of p75 IL-2R on NK cells. Peripheral blood leukocytes were stained with FITC-conjugated IgG2a control mAb and control purified IgG1 mAb or FITC conjugated anti-Leu-11d (CD16) (IgG2a mAb) and purified anti-p75 IL-2R (IgG1 mAb). PE-conjugated rat anti-mouse IgG1 mAb was used to detect anti-p75 IL-2R mAb. Samples were analyzed using a FACScan. Based on characteristic forward and wide angle light scatter patterns, electronic gates were set on lymphocytes, monocytes, and granulocytes, as indicated. (*A*) Histograms of anti-p75 IL-2R–stained cells were superimposed over histograms of control Ig stained cells (histograms nearest the ordinate). (*B*) Contour plots of lymphocytes stained with fluorochrome-conjugated control Ig or FITC anti-CD16 and anti-p75 IL-2R, followed by PE conjugated anti-mouse IgG1. Markers defining quadrants were positioned to include >98% of the control IgG stained cells in the lower left quadrant.

NK cells with rIL-2 in vitro results in the induction of activation antigens, the acquisition of cytotoxic activity against NK-resistant tumors (i.e., LAK activity), and proliferation (8, 9, 14, 16). An increased number and proportion of circulating NK cells expressing activation antigens and enhanced cytolytic activity have also been noted in patients treated with rIL-2 in vivo (17). Based on indirect evidence, it has been suggested that the effect of IL-2 on NK cells is mediated through the p75 IL-2R (10, 11). However, in addition to the 75-kD IL-2R, a 70-kD protein is also detected by crosslinking ^{125}I-IL-2 to the membrane of NK cells (10, 11) and other membrane structures, e.g., HLA (18) and OKT27 (19), have been shown to be associated with IL-2 binding. Therefore, further studies were undertaken to determine the effect of anti-p75 IL-2R mAb on activation of NK cells by IL-2.

Resting, peripheral blood low buoyant density lymphocytes, enriched for NK cells, (Fig. 2, *A*, *B*) or FACS-purified CD3$^-$CD16$^+$ NK cells (Fig. 2, *C*, *D*) were cultured

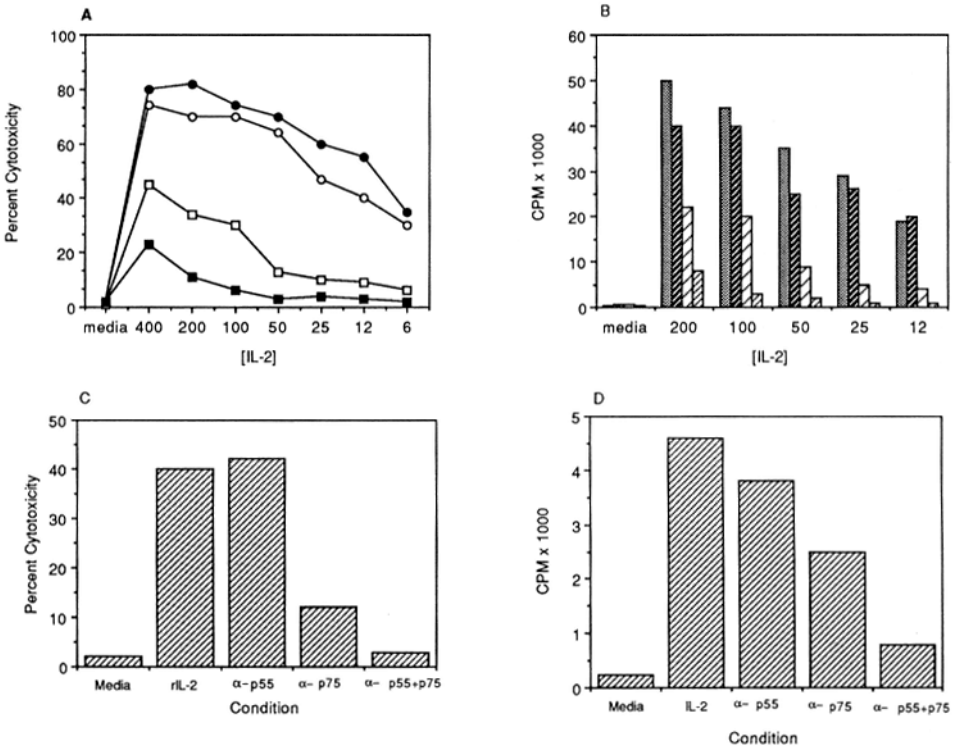

FIGURE 2. Effects of anti-IL-2R mAbs on NK cells cytotoxicity and proliferation. (*A*, *B*) Low buoyant density peripheral blood lymphocytes, enriched for NK cells (~40% CD3$^-$, CD16$^+$), were cultured in various concentrations of rIL-2 in the presence or absence of isotype-matched control Ig (*A*, ●; *B*, ▨), anti-p55 IL2-R(*A*, ○; *B*, ■), anti-p75 IL-2R (*A*, □; *B*, ▨), or both anti-p55 IL-2R and anti-P75 IL2-R (*A*, ■; *B*, ▨) (mAb concentration, 10 μg/ml). (*C*, *D*) FACS purified (>97%) CD3$^-$,CD16$^+$ NK cells were cultured with 200 U/ml rIL-2 in the presence or absence of isotype-matched control Ig, anti-p55 IL2-R and/or anti-p75 IL-2R (mAb concentration, 10 μg/ml), as indicated. Cytotoxicity against the NK-resistant Colo-205 cell lines was assayed after 18 h culture (*A*, *C*), and proliferation was assessed after 4 d (*B*, *D*). For proliferation assays, cells were plated at 5 × 10^6/ml (*B*) or 5 × 10^4 cells/ml (*D*).

with rIL-2 in the presence or absence of anti-p75 IL-2R and/or anti-p55 IL-2R mAb. As shown in Fig. 2 *A*, anti-p75 IL-2R substantially inhibited the IL-2-induced cytotoxicity against an NK-resistant target, with maximal inhibition at lower concentrations of IL-2. Anti-p55 IL-2R alone had no affect on IL-2-induced cytotoxicity, as reported previously (7, 8). However, combining both anti-p55 IL-2R and anti-p75 IL-2R mAb did inhibit induction of cytotoxicity more efficiently than anti-p75 IL-2R alone. Thus, although no p55 IL-2R was detected on the $CD16^+$ NK population by flow cytometry, low levels of this receptor may be present and functionally active. Neither anti-p75 IL-2R nor anti-p55 IL-2R mAb affected cytotoxicity against K562 mediated by unstimulated NK cells (not shown). Consistent results were observed using NK cells from 5 donors. IL-2 induced proliferation of low buoyant density lymphocytes (Fig. 2 *C*) and FACS purified NK cells (Fig. 2 *D*) was partially inhibited by anti-p75 IL-2R or anti-p55 IL-2R mAb alone, but maximal inhibition was observed by combining both antibodies. Culture of NK cells in rIL-2 induces expression of p55 IL-2R on NK cells, resulting in the formation of high affinity IL-2R (10, 16).

Stimulation of NK cells with IL-2 results in the rapid induction of a newly synthesized cell surface activation antigen, Leu-23 (CD69) (9). Leu-23 is a disulfide-linked homodimer that is phosphorylated and expressed within a few hours after lymphocyte activation (9). >90% of NK cells express Leu-23 after exposure to IL-2, indicating that these cells are functionally responsive to this signal. To determine whether the IL-2-mediated induction of Leu-23 is transmitted through the p75 IL-2R, resting NK cells were cultured in IL-2 in the presence or absence of anti-p75 IL-2R or anti-p55 IL-2R mAb. As shown in Fig. 3, induction of Leu-23 was completely inhibited by anti-p75 IL-2R, but was unaffected by anti-p55 IL-2R. In summary, results from the present studies directly implicate the p75 IL-2R as the structure predominantly responsible for IL-2 activation of NK cells.

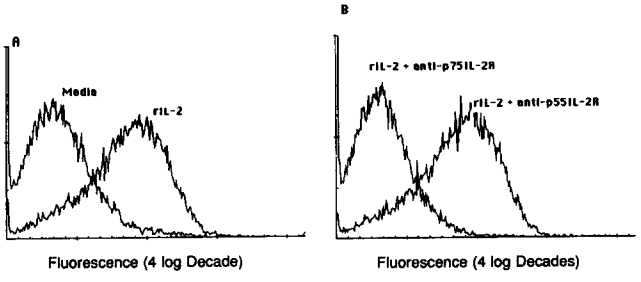

FIGURE 3. Inhibition of CD69 induction. Low buoyant density peripheral blood lymphocytes, enriched for NK cells (~40% $CD3^-$, $CD16^+$), were cultured for 18 h with or without 200 U/ml rIL-2 in the absence or presence of isotype-matched control Ig, anti-p55 IL-2R or anti-p75 IL-2R (mAB concentration, 10 μg/ml). Cells were harvested and stained with PE-conjugated anti-Leu-23 (CD69) mAb and FITC-conjugated anti-Leu-11a (CD16). An electronic gate was set on $CD16^+$ (i.e., FITC stained) cells and data were reprocessed to determine the amount of CD69 expressed on the NK cells. (*A*) Histograms of CD69 fluorescence from NK cells cultured overnight in "medium" or "rIL-2" with IgG1 control mAb. Histograms of cells stained with PE-conjugated control IgG1 mAb were identical to the "medium" histogram (not shown). (*B*) Histograms of CD69 fluorescence from NK cells cultured overnight in rIL-2 with anti-p75 IL-2R or with anti-p55 IL-2R mAb. Histograms of cells stained with PE-conjugated control IgG1 mAb were identical to the "rIL-2 + anti-p75" histogram (not shown).

Summary

The IL-2R is composed of at least two subunits, the p55 (CD25/Tac) and p75 glycoproteins. The p75 IL-2R is expressed preferentially on resting human peripheral blood NK cells, but is not detected on substantial proportions of T and B lymphocytes, monocytes, or granulocytes. Anti-p75 IL-2R mAb substantially inhibits the early events associated with NK cell activation by IL-2, including inhibition of cytotoxic activity and induction of the CD69 early activation antigen. While anti-p55 IL-2R mAb alone failed to substantially inhibit the initial events of IL-2 stimulation, maximal inhibition of IL-2-induced cytotoxicity and proliferation was achieved by combining both anti-p55 IL-2R and anti-p75 IL-2R. Collectively, results from the present studies directly implicate the p75 IL-2R as the structure predominantly responsible for IL-2 activation of NK cells.

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