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CONSTITUTIVE EXPRESSION OF HIGH AFFINITY INTERLEUKIN 2 RECEPTORS ON HUMAN CD16$^-$ NATURAL KILLER CELLS IN VIVO

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IL-2, a lymphokine secreted by T cells that functions in the growth and differentiation of lymphocytes and monocytes, binds to a 55-kD glycoprotein (p55), designated CD25, with low affinity (~10–40 nM kD) and a 70–75 kD glycoprotein (p75) with an intermediate affinity (~1–5 nM kD) (1–4). The high affinity IL-2 receptor (~10–30 pM kD) is composed of noncovalently associated p55 and p75 subunits (1–4). Several laboratories have generated mAbs against the p55 and p75 IL-2 binding proteins (5, 6), thus allowing for the detection of these receptors on leukocyte populations.

NK cells are a distinct population of CD3ϵ^-,CD56$^+$ lymphocytes that do not rearrange or express TCR genes and are characterized by their ability to mediate MHC-unrestricted cytotoxicity against a variety of tumor- and virus-infected cell lines (7). IL-2 directly activates NK cells, resulting in proliferation and the induction of cytotoxic activity (8–10). The majority of peripheral blood CD16$^+$ NK cells constitutively express the p75 IL-2R, and mAbs against p75 IL-2R efficiently block IL-2-dependent functions, suggesting that the p75 IL-2R is the predominant IL-2 binding structure on resting NK cells (11). We have previously reported that two subsets of NK cells can be identified based on expression of CD16 (FcRγIII). Whereas most peripheral blood NK cells express the phenotype CD16$^+$,CD56$^+$, a minor subset of NK cells are CD16$^-$,CD56$^+$ (referred to as CD16$^-$ NK cells) (12, 13). Neither of these subsets expresses CD3ϵ or rearranges TCR genes, but both mediate NK cytotoxic function and express many similar phenotypic characteristics (12, 13). In the present study, we demonstrate that CD16$^-$ NK cells constitutively express high affinity IL-2 receptors in vivo and preferentially respond to low amounts of IL-2.

Materials and Methods

Cell Purification and Culture. PBMC were isolated by Ficoll/Hypaque centrifugation. After plastic adherence and passage through nylon wool, NK cells were enriched by centrifugation on discontinuous gradients of Percoll (Pharmacia Fine Chemicals, Piscataway, NJ) (10). Cells were cultured in RPMI 1640 media (M. A. Bioproducts, Walkersville, MD) supplemented with 4% horse serum (KC Biologicals, Lenexa, KS), 1 mM L-glutamine (Gibco Laboratories, Grand Island, NY), and antibiotics. rIL-2 (18×10^6 IU/mg) was generously provided by Cetus Corp., Emeryville, CA. For proliferation assays, cells were incubated in 96-well cul-

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ture plates and harvested after 4 d following an overnight pulse with [^{3}H]thymidine (1 μCi/well; Amersham Corp., Arlington Heights, IL).

Monoclonal Antibodies. TU27 mAb (generously provided by Dr. K. Sugamura, Tohoku University School of Medicine, Sendai, Japan) reacts with the p75 IL-2R and inhibits binding of IL-2 to this receptor (5). Leu series mAbs and anti-CD25 (clone 2A3) were produced by Becton Dickinson Monoclonal Center, Inc. (Mountain View, CA). Phycoerythrin (PE)-conjugated TU27 and anti-CD25 mAbs were generously provided by Dr. A. Jackson and Mr. A. Blidy, Becton Dickinson Monoclonal Center.

Immunofluorescence and Flow Cytometry. Immunofluorescence and flow cytometry were performed as previously described (10). Cells were analyzed on a FACScan, and cell sorting was performed on a FACStar or FACS IV (Becton Dickinson Immunocytometry Systems, San Jose, CA).

Cytotoxicity Assay. Cell-mediated cytotoxicity was measured in a 4-h ^{51}Cr-release assay (10).

IL-2 Binding Assay. IL-2 binding assays and Scatchard analysis were performed as previously described (14). All reagents for the binding assays were generously provided by Dr. Kendall Smith (Dartmouth Medical School, Hanover, NH).

Results and Discussion

Expression of p75 and p55 IL-2 Receptors on CD16$^-$ and CD16$^+$ NK Cells. PBL enriched for NK cells using Percoll density gradients were stained with FITC anti-CD56 and PE anti-CD25 or PE anti-p75 IL-2R and analyzed by two-color immunofluorescence. As previously reported (12), the majority of circulating resting NK cells express low surface density CD56 and high surface density CD16 (CD16$^+$ CD56^{dim+} NK cells). A small subset of NK cells do not express CD16, but have significantly higher surface density CD56 (CD16$^-$CD56$^{bright+}$ NK cells). Neither of these NK cell subsets express CD3 (12). Based on analysis of more than 20 normal donors, both CD16$^-$ CD56^{bright} and CD16$^+$CD56^{dim+} NK cell subsets expressed the p75 IL-2R; however, only the CD16$^-$CD56$^{bright+}$ NK cells constitutively expressed detectable CD25 (Fig. 1). The finding that the major subset of NK cells (CD16$^+$-CD56^{dim+}) express only the p75 IL-2R and mediate IL-2-dependent functions primarily through an intermediate affinity receptor is consistent with prior reports (1–4, 11). Co-expression of CD25 and p75 IL-2R on the CD16$^-$CD56$^{bright+}$ NK cells suggested that these cells possess high affinity IL-2 receptors.

Differential response of CD16$^-$ and CD16$^+$ NK Cells to Low Concentrations of rIL-2. CD16$^-$ and CD16$^+$ NK cells were isolated to >98% purity by cell sorting, incubated with various concentrations of rIL-2, and assayed for cytolytic activation and IL-2-dependent proliferation (Fig. 2). Consistent with the presence of high affinity IL-2 receptors, CD16$^-$ NK cells showed a significantly greater magnitude of IL-2-induced cytolytic activation and proliferation than CD16$^+$ NK cells. CD16$^-$ NK cells demonstrated potent cytolytic activation and proliferation at IL-2 concentrations as low as 9 IU/ml. CD16$^+$ NK cells required significantly higher concentrations of IL-2, consistent with intermediate affinity IL-2 receptors being the primary IL-2 signal-transducing structure on these cells.

IL-2 Binding Assays. IL-2 binding assays were performed to determine the number and affinity of the IL-2 receptors. Scatchard analysis demonstrated the presence of ~1,200 high affinity (~25 pM kD) and ~9,600 intermediate affinity (~2 nM kD) IL-2 receptors on CD16$^-$ NK cells (Fig. 3). CD16$^+$ NK cells expressed only a single intermediate affinity IL-2 receptor of ~1.9 nM kD (~9,000 sites per cell). The IL-2 binding data thus substantiated the phenotypic and functional studies and defini-

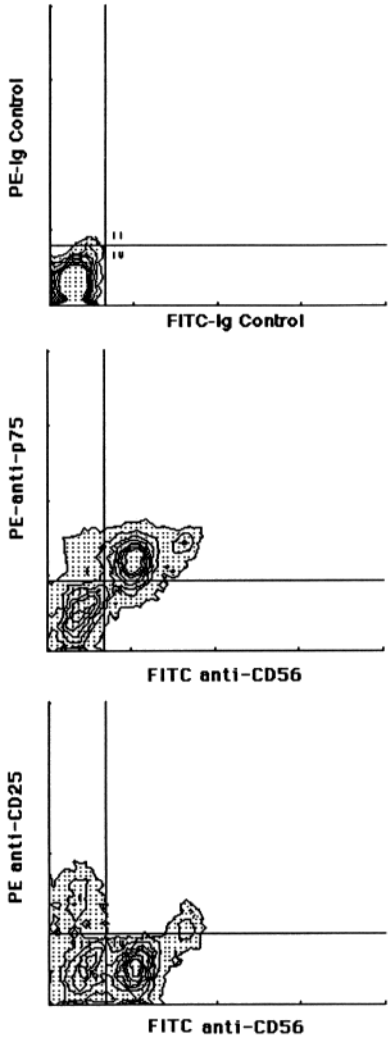

FIGURE 1. Expression of IL-2 receptors on NK cell subsets. PBL enriched for NK cells were stained with FITC-conjugated IgM control mAbs and PE-conjugated IgG1 control mAb (*A*) or FITC-conjugated anti-CD56 (clone L185) and PE-conjugated anti-p75 IL-2R (TU27; *B*) or FITC-conjugated anti-CD56 and PE-conjugated anti-CD25 (clone 2A3; *C*). Flow cytometric data are presented as contour plots (*x* and *y* axes are 4-decade log scales). Markers defining quadrants were positioned to include >98% of unstained cells in lower left quadrant.

tively showed that the differential responsiveness of $CD16^-$ and $CD16^+$ NK cells to IL-2 is manifested through different affinity IL-2 receptors.

Effects of Anti-IL-2 Receptor mAbs on IL-2-dependent Functions. Experiments were undertaken to determine the effects of blocking mAbs against p75 IL-2R and CD25 on IL-2-dependent functions of $CD16^-$ and $CD16^+$ NK cell subsets. As previously reported (11), mAbs against p75 IL-2R effectively blocked IL-2 activation of $CD16^+$ NK cells (Fig. 4, *A*, *B*). Interestingly, although anti-CD25 mAb alone failed to affect IL-2-induced activation (as would be expected from the phenotypic and binding studies), anti-CD25 mAb slightly increased the inhibition observed with anti-p75 IL-2R mAb (Fig. 4, *A*, *B*). These results suggest that functional high affinity receptors may be present in very low levels (undetectable by IL-2 binding assays or flow cytom-

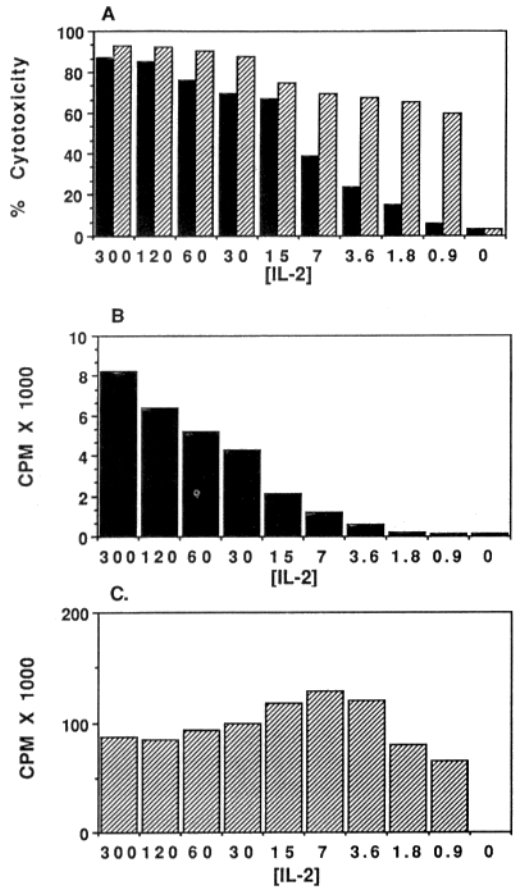

FIGURE 2. Effects of IL-2 concentration on activation and proliferation of NK cell subsets. $CD16^{+}CD56^{dim}$ (■) and $CD16^{-}CD56^{bright}$ (▨) NK cells were sorted to >98% purity, incubated with various concentrations of IL-2 (IU/ml × 10^{-1}) overnight, and assayed for cytotoxicity against the NK cell-resistant tumor cell line Colo-205 at an E/T ratio of 25:1 (*A*). Resting NK cell subsets do not lyse Colo-205 (10). For proliferation assays (*B*, *C*), cells were plated at 5 × 10^6 cells/ml (*B*) or 5 × 10^4 cells/ml (*C*) in the presence of various concentrations of IL-2. Proliferation was assessed by [^{3}H]thymidine incorporation after 4 d in culture.

etry) on these cells and that neutralization requires the presence of both anti-p75 IL-2R and anti-CD25 mAb.

IL-2-induced cytolytic activation of $CD16^-$ NK cells was inhibited by the anti-p75 IL-2R mAb, but not by anti-CD25 mAb (Fig. 4 *A*). A combination of both anti-p75 IL-2R and anti-CD25 mAbs was more effective than anti-p75 IL-2R mAb alone in inhibiting cytolytic activation. Surprisingly, neither anti-p75 IL-2R nor anti-CD25 mAb alone affected IL-2-dependent proliferation of $CD16^-$ NK cells, whereas a combination of both mAb gave pronounced inhibition (Fig. 4 *C*). Previous studies have shown that these mAbs against CD25 and p75 IL-2R are effective in inhibiting binding of IL-2 to the high affinity receptor (5). Since $CD16^-$ NK cells express high affinity IL-2 receptors presumably consisting of a p75/p55 complex, it was surprising that high concentrations (20–30 μg/ml) of these mAbs individually had no significant effect on IL-2-induced proliferation. Similar results have recently been reported for mAb blocking of IL-2-dependent proliferation of T cell lines expressing both intermediate and high affinity IL-2 receptors (5, 6). Relative affinity and binding kinetics of the mAb versus ligand may account for these results.

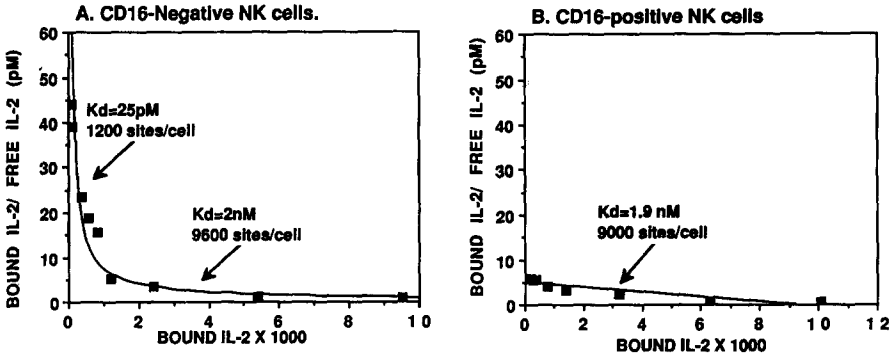

FIGURE 3. Binding of [^{125}I]IL-2 to NK cell subsets. Peripheral blood CD16$^-$ (*A*) and CD16$^+$ (*B*) NK cells, isolated to >98% purity, were incubated with serial various concentrations of ^{125}I-labeled IL-2 for 45 min at 37°C (2×10^5 cells/determination) and then centrifuged through a cushion of oil to separate bound from unbound IL-2 (14). Nonspecific binding was determined in the presence of 200 molar excess of unlabeled IL-2. Data are represented as Scatchard plots.

Recently, it has been shown that a substantial proportion of peripheral blood CD4$^+$ T lymphocytes express low levels of CD25 (15); however, these T cells usually express only low affinity IL-2 receptors, lack p75 IL-2R, and are unresponsive to IL-2 (5, 6, 11, and Phillips, J. H., unpublished observation). Therefore, the CD16$^-$ NK cell subset represents the only consistently detectable population of lymphocytes in peripheral blood of normal adults that constitutively expresses high affinity IL-2 receptors in vivo. Based on an extensive study of these cells, we have suggested that CD16$^-$ NK cells may represent a peripheral blood progenitor population for mature CD16$^+$ NK cells (13). If this is the case, the presence of high affinity IL-2 receptors may allow expansion and differentiation of these cells with physiological concentrations of IL-2. Alternatively, CD16$^-$ NK cells may represent a distinct NK subset that constitutively expresses high affinity IL-2 receptors as a consequence of activation in vivo. Nonetheless, the presence of high affinity IL-2 receptors would permit preferential responsiveness of CD16$^-$ NK cells in patients treated with rIL-2 for therapeutic purposes. Recent studies have shown a pronounced expansion of the CD16$^-$ NK cell subset in the peripheral blood of cancer patients after in vivo rIL-2 therapy (16, 17). During normal immune responses, CD16$^-$ NK cells may respond to IL-2 produced in vivo by T cells after antigenic stimulation. CD16$^-$ NK cells have been shown to transcribe TNF-α and granulocyte/macrophage CSF (GM-CSF) after activation with IL-2 in vitro (13). If this occurs in vivo, these cells may be an important source of cytokine production in physiological situations.

Summary

The majority of human NK cells express low affinity IgG Fc receptors (CD16$^+$), whereas a minor subset of NK cells lack Fc receptor expression (CD16$^-$). In contrast to CD16$^+$ NK cells that express only p75 IL-2 receptors, CD16$^-$ NK cells constitutively co-express both p75 and p55 IL-2 receptors in vivo and preferentially respond to low concentrations of IL-2 with increased cytolytic activation and prolifer-

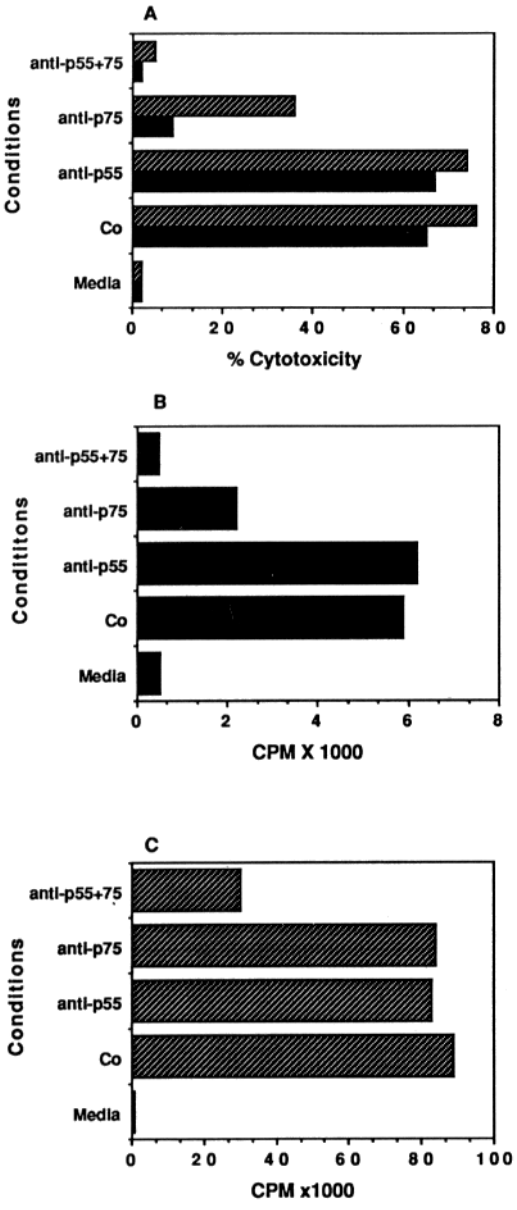

FIGURE 4. Effects of anti-IL-2R mAbs on IL-2-induced cytotoxicity and proliferation. $CD16^+$ (■) and $CD16^-$ (▨) NK cell subsets were isolated to >98% purity by cell sorting and incubated with # IU/ml rIL-2 in the presence or absence of control (Co) or neutralizing anti-p75 and/or p55 (CD25) IL-2R mAb (30 μg/ml), as indicated. Cells were harvested after overnight culture for cytotoxicity assays (target # Colo-205; E/T ratio, 25:1). Proliferation was assessed by [^{3}H]thymidine incorporation after 4 d in culture.

ation. Scatchard analysis demonstrated the presence of ~1,200 high affinity (~25 pM kD) and ~9,600 intermediate affinity (~2 nM kD) IL-2 receptors on $CD16^-$ NK cells. $CD16^+$ NK cells expressed only a single intermediate affinity IL-2 receptor of ~1.9 nM kD (~9,000 sites per cell). The IL-2 binding data thus substantiated the phenotypic and functional studies and definitively show that the differential responsiveness of $CD16^-$ and $CD16^+$ NK cells to IL-2 is manifested through different affinity IL-2 receptors.

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