Novel Interleukin 2 (IL-2) Receptor Appears to Mediate IL-2–induced Activation of Natural Killer Cells

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Abstract

A novel IL-2 receptor, distinct from the Tac protein, has been identified on the surface of purified human natural killer (NK) cells by chemical cross-linking of ^{125}I-IL-2. This protein is ~ 70,000 D in size (p70) and appears to be identical to the recently recognized second subunit of the human high affinity IL-2 receptor complex. Scatchard analysis of ^{125}I-IL-2 binding to purified NK cells revealed ~ 2,300 p70 binding sites per cell with an apparent dissociation constant of 200 pM, a value intermediate between the previously recognized high and low affinity forms of the human IL-2 receptor. The monoclonal anti-Tac antibody did not inhibit the cross-linking of ^{125}I-IL-2 to the p70 binding sites present on NK cells. Functionally, the addition of high concentrations of recombinant IL-2 to the enriched NK cells promoted a rapid augmentation of cytolytic activity and a more delayed increase in cellular proliferation. Anti-Tac effectively blocked the IL-2–induced proliferative response in these cells, but failed to alter the enhancement of cytotoxicity. Analysis of NK cytoplasmic RNA isolated at various time points after IL-2 stimulation revealed the rapid induction of c-myb and Tac gene expression that was also not inhibited by the anti-Tac antibody. These findings suggest that IL-2 binding to the p70 receptor constitutively expressed on the surface of NK cells may mediate both the development of increased cytolytic activity and rapid changes in gene expression. The activation of the Tac gene may in turn permit the formation of the high affinity IL-2 receptor complex (comprised of at least the Tac and p70 proteins) that appears to transduce the requisite signals involved in NK cell proliferation.

Introduction

Natural killer (NK)[1] cells represent a heterogeneous population of lymphoid cells that exhibit cytotoxic activity against certain tumors and virally infected cells (reviewed in references 1 and 2). Characteristically, these cells are large and granular in morphology (3), react with the Leu 11 antibody (4, 5), and exhibit cytolytic function that is unrestricted by elements of the major histocompatibility complex (1, 2). The level of cytotoxic activity in peripheral blood lymphocytes and purified Leu 11^+ cell populations can be amplified by the addition of IL-2 (6–10). The IL-2–induced changes in NK cell cytotoxicity are (*i*) detectable within 4 h (9), (*ii*) dependent on protein but not DNA synthesis (9), (*iii*) dependent on relatively high concentrations of IL-2 (100–500 U/ml) (8–10), and (*iv*) independent of Tac antigen expression. Furthermore, purified Leu 11^+ cell populations often express negligible levels of the Tac antigen, and Il-2–induced augmentation of their cytolytic activity is not inhibited by the anti-Tac antibody (8–11).

Recent ^{125}I-IL-2 cross-linking studies with various human lymphoid cell lines and activated normal human T cells have led to the identification of a second human IL-2 binding protein. This protein is ~ 70,000–75,000 D in size (p70), and appears able to noncovalently associate with the Tac antigen, forming a membrane complex that binds IL-2 with high affinity (12–16). Expression of the p70 protein in the absence of the Tac antigen has been detected on certain lymphoid cell lines, including MLA-144 T cells (13–16), SKW 6.4 B cells (16), and "natural killer like" YT cells (15, 16) as well as on various normal lymphoid populations, including resting T cells (16, 17) and large granular lymphocytes (16).

In the present study, we have further characterized and quantitated the p70 IL-2 binding proteins present on human NK cells and investigated the potential functional role of these receptors in mediating IL-2–induced changes in NK cell cytotoxicity and proliferation.

Methods

Cell preparation. Human PBMC were obtained by leukopheresis of normal donors and separated from erthrocytes by centrifugation on Hypaque–Ficoll. The mononuclear cells were passed over nylon wool columns to deplete B cells and monocytes (18). The Leu 11^+ cells were further purified by negative selection utilizing an affinity rosetting technique (19). Briefly, the cells were incubated with a cocktail of monoclonal antibodies (anti-Leu 1 and anti-DR; Becton Dickinson & Co., Sunnyvale, CA), (OKT3; Ortho Diagnostic Systems Inc., Raritan, NJ), washed, and mixed with oxred blood cells previously coated with goat $F(ab')_2$ anti-mouse Ig (Boehringer Mannheim Biochemical, Indianapolis, IN) using a chromic chloride method. Cells forming rosettes with the ox-coated erythrocytes were eliminated from the suspensions by centrifugation on Hypaque–Ficoll cushions. These negatively selected NK cells were routinely 90–95% Leu 11^+ and < 2% OKT3 positive, as determined by indirect immunofluorescent staining with the anti-Leu 11a (Becton Dickinson & Co.) antibody.

Reagents. Recombinant IL-2 used in this study was kindly provided by the Cetus Corporation (Emeryville, CA). This material was 99% pure by SDS-PAGE analysis and contained < 0.01 ng endotoxin per 10^6 U (20). 1 pmol of IL-2 per liter (1 pM) is equivalent to 0.2 Biological Response Modifier Program (BRMP) reference units per ml. The anti-Tac antibody used has been previously described (21, 22). The UPC10 monoclonal antibody, an IgG_{2a} myeloma protein of

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1. *Abbreviations used in this paper:* BRMP, Biological Response Modifier Program; NK, natural killer.

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unknown specificity that is isotyped matched to the Tac protein, was purchased from Sigma Chemical Co. (St. Louis, MO).

Measurement of DNA synthesis. 1×10^5 cells were plated in 200 μl of RPMI 1640 plus 10% FCS and 1 μCi of [^{3}H]thymidine (New England Nuclear, Boston, MA) was added during the final 18 h of culture. The amount [^{3}H]thymidine incorporated was measured by standard liquid scintillation techniques. To examine the effects of anti-Tac on IL-2–induced DNA synthesis, various concentrations of purified anti-Tac antibody were added at the initiation of cultures.

Cytotoxicity assay. NK activity was evaluated in a 4-h ^{51}Cr release cytotoxicity assay using NK sensitive K562 cells as targets. Effector cells were incubated for 18 h in the presence of various concentrations of recombinant IL-2 in RPMI 1640 plus 10% FCS. In some experiments purified anti-Tac antibody was added. Target cells were labeled for 1 h with 300 μCi of ^{51}Cr (ICN Radiochemicals, Irvine, CA), washed three times, and resuspended in RPMI 1640 with 10% FCS. 10,000 labeled target cells (100 μl) were mixed with varying numbers of effector cells (100 μl) in 96-well V-bottom microtiter plates (Costar, Cambridge, MA). Spontaneous release of ^{51}Cr by target cells was determined by placing labeled target cells in microtiter wells in the absence of effector cells. Cultures were incubated in 5% CO_2 at 37°C in 100% humidity for 4 h. Plates were centrifuged and 100 μl of supernatant removed and counted in a γ-counter. Percent cytotoxicity was determined by the following formula: (mean experimental cpm) − (spontaneous release cpm)/(maximal release cpm) − (spontaneous release cpm) × 100. In all experiments spontaneous ^{51}Cr release was < 10%.

^{125}I-IL-2 chemical cross-linking. ^{125}I-IL-2 (37.1 μCi/μg; New England Nuclear) was allowed to bind to cells at a final concentration of 5 nM (low affinity) in the presence or absence of a 200-fold molar excess of UPC10, anti-Tac, or unlabeled recombinant IL-2. The binding was performed at 4°C using 20×10^6 Leu 11$^+$ lymphocytes or 10×10^6 HUT 102B2 cells suspended in 1 ml of RPMI 1640 supplemented with 2% FCS and 25 mM Hepes (pH 7.4). After 2 h of incubation the cells were pelleted; resuspended in 1 ml of PBS (pH 8.3) containing 1 mM $MgCl_2$ and 0.2% azide, and cross-linked by the addition of noncleavable cross-linker disuccinimidyl suberate (100 μg/ml final concentrations; Pierce Chemical Co., Rockford, IL). The cells were incubated for 30 min at room temperature, washed three times with PBS, and broken by dounce homogenization. The nuclei were removed by centrifugation at 400 *g* and the membrane-enriched fraction was recovered from the supernatant by centrifugation at 100,000 *g* for 1 h. The membranes were solubilized in SDS sample buffer, boiled for 3 min, and analyzed by SDS-PAGE on 7.5% gels under reducing conditions.

^{125}I-IL-2 radioreceptor binding assays. As previously described (23, 24), 2.5×10^6 Leu 11$^+$ cells were suspended in 100 μl of binding buffer (RPMI 1640 containing 2% FCS, 0.1% sodium azide, 25 mM Hepes, pH 7.4) and incubated with increasing concentrations of ^{125}I-IL-2 in the presence or absence of a 500-fold molar excess of unlabeled IL-2 and 200-fold molar excess of purified anti-Tac antibody. After a 2-h incubation at 4°C, cell associated and free ^{125}I-IL-2 were separated by overlaying the cell suspension on 200-μl cushions of oil (84% silicon oil; Dexter Hysol, Olean, NY, and 16% paraffin oil; Fisher Scientific Co., Pittsburgh, PA) and centrifugation at 12,000 *g* for 1 min. Radioactivity present in the supernatant and excised tips were measured. The resulting data were analyzed by Scatchard plots and receptor number and apparent binding affinity calculated.

Cytoplasmic dot blots. IL-2–induced changes in specific mRNA levels in Leu 11$^+$ cells were analyzed by cytoplasmic dot hybridization as previously described (25). The nitrocellulose filters were baked and hybridized with varying cDNA probes radiolabeled with ^{32}P by the random priming method of Feinberg (26). Conditions of hybridization and washing were as previously described (27).

Results

To confirm the relative purity of the NK cell preparations, indirect immunofluorescent staining and flow microfluorometric analyses were performed. As shown in Fig. 1 *A*, > 90% of these cells specifically reacted with the Leu 11a monoclonal antibody, a cell surface marker for human NK cells (4). Also, these enriched NK cells for the most part lacked expression of the Tac antigen as < 4% of the cell population weakly reacted with the anti-Tac antibody (Fig. 1 *B*).

In agreement with previous studies (6–10), IL-2 produced a dose-related enhancement of the cytotoxic activity of these purified NK cells against NK-sensitive K562 target cells (Fig. 2). Although some increase in cytolytic activity was observed after the addition of relatively low doses of IL-2 (10 pM), greater stimulation was always observed at higher concentrations of this lymphokine (10 nM). Inclusion of a 200-fold molar excess of the anti-Tac antibody relative to the IL-2 concentration did not alter the IL-2–mediated augmentation of cytolytic activity (Fig. 3 *A*).

In addition to enhancing cytotoxicity, IL-2 also stimulated an increase in NK cell proliferation as measured by [^{3}H]-thymidine incorporation (Fig. 3 *B*). However, in contrast to the inability of anti-Tac to block changes in cytolytic function, this monoclonal antibody almost completely inhibited IL-2–induced changes in NK cell proliferation (Fig. 3 *B*).

Taken together, these results suggest that the Tac antigen plays a critical role in IL-2–induced NK cell proliferation, but is not required for IL-2–induced enhancement of NK cytotoxic activity. To explore the possible existence of an IL-2 binding protein distinct from the Tac antigen, which potentially mediates IL-2–induced changes in NK cytotoxicity, ^{125}I-IL-2 chemical cross-linking studies were performed. Under conditions permitting detection of both high and low affinity IL-2 receptors (5 nM final concentration of ^{125}I-IL-2) two major cross-linked bands of 68,000 D (band *A*) and 85,000 D (band *B*) were detected on HTLV-I–infected HUT 102B2 cells, a T cell line that displays both high and low affinity IL-2 receptors (Fig. 4, lane *4*). Previous studies have demonstrated that these two protein bands correspond to ^{125}I-IL-2 cross-linked to the 50,000–55,000-D Tac antigen (band *A*) and a 70,000–75,000-D polypeptide (band *B*, p70). Also, these investigations have shown that the Tac and p70 proteins are able to independently bind IL-2 with low (dissociation constant of [K_d] of 20–30 nM) or intermediate affinity (K_d of 0.6–1.2 nM), respectively. Furthermore, when associated, these proteins appear to form a membrane receptor complex that binds IL-2 with high affinity (12–16). In contrast to HUT 102B2 cells, cross-linking of ^{125}I-IL-2 to the NK cell population revealed only the presence of band *B* indicating expression of the p70 protein in the absence of the Tac antigen (Fig. 4, lane *2*). The

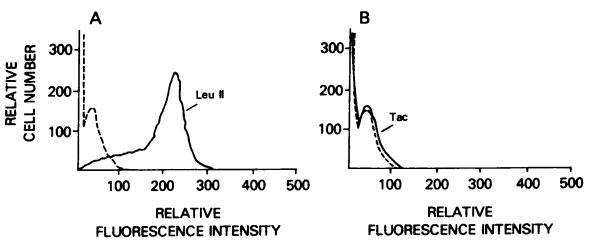

Figure 1. Fluorescent staining of purified NK cells. 1×10^6 negatively selected cells were immunofluorescently stained with either anti-Tac (– – –) (*A*) leu 11a (---) (*B*) or an isotype-matched control (– – –) antibody and analyzed on a FACS.

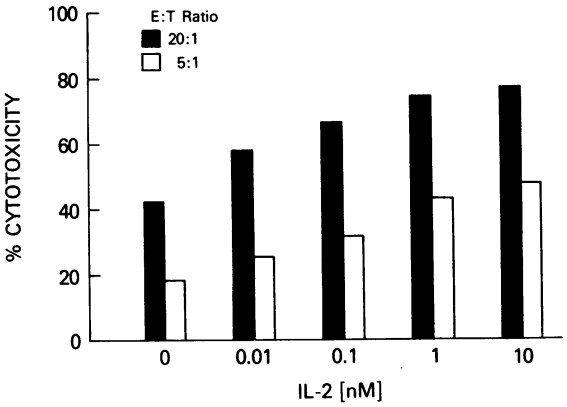

Figure 2. Increased NK cytolytic activity in response to IL-2. Leu 11^+ lymphocytes were purified from peripheral blood as detailed in the Methods and cultured for 18 h in the presence or absence of various concentrations of recombinant IL-2. The cells were washed twice and then assessed for NK cytolytic activity in a 4-h ^{51}Cr-release assay. 1 nM concentration IL-2 is equivalent to 200 BRMP reference units per ml.

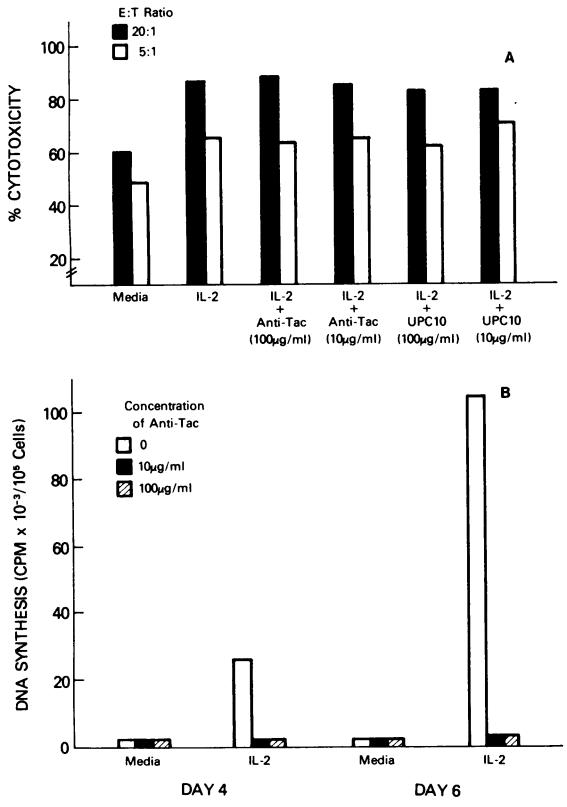

Figure 3. Anti-Tac does not inhibit IL-2–induced augmentation of NK cytolytic activity, but markedly inhibits IL-2–induced proliferation. (*A*) Leu 11^+ lymphocytes were cultured for 18 h in the presence of IL-2 (5 nM) and in the presence of either 100 μg/ml of anti–Tac antibody or 100 μg/ml of the UPC10 antibody. The cells were washed twice and then assessed for NK cytolytic activity in a 4-h ^{51}Cr-release assay. (*B*) Leu 11^+ lymphocytes were cultured for 4 or 6 d in the presence or absence of the anti-Tac (100 μg/ml) with media alone or a 5 nM concentration of recombinant IL-2. [^{3}H]Thymidine incorporation was measured over the last 18 h of the culture period. 5 nM concentration of IL-2 is equivalent to 1,000 BRMP reference units per ml.

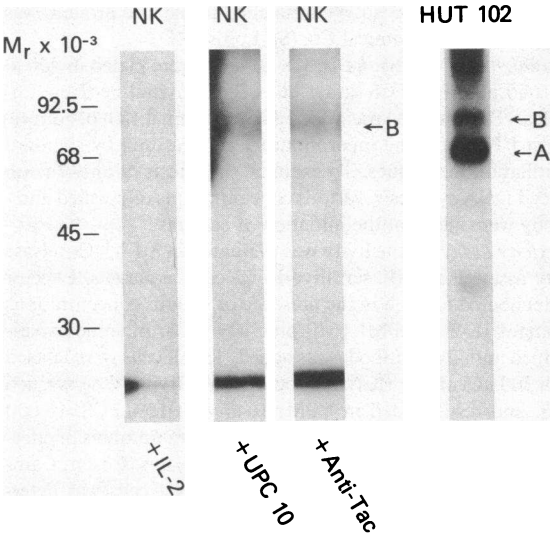

Figure 4. ^{125}I-IL-2 cross-linking to NK cells detects an IL-2 binding protein distinct from Tac. ^{125}I-IL-2 was allowed to bind to purified Leu 11^+ cells and to Hut 102 B2 cells under low affinity (5 nM) binding conditions. The bound ^{125}I-IL-2 was chemically cross-linked by the addition of dissuccinimidyl suberate as described in the Methods. Proteins were analyzed by SDS-PAGE using 7.5% gels under reducing conditions.

cross-linking to the p70 protein reflected specific binding of the radiolabeled lymphokine since the addition of a 200-fold molar excess of unlabeled IL-2 blocked detection of this cross-linked band (Fig. 4, lane *1*). In contrast, addition of similar molar excess of the anti–Tac or control UPC10 monoclonal antibodies did not alter cross-linking of ^{125}I-IL-2 to this receptor protein (Fig. 4, lanes *2* and *3*). These findings suggest the constitutive expression of the p70 IL-2 binding protein in the absence of the Tac antigen on the surface of freshly isolated and purified NK cells.

To quantitate the number and affinity of the p70 IL-2 binding sites on these NK cells, ^{125}I-IL-2 binding assays were performed. These studies were conducted in the presence of the anti–Tac antibody (100 μg/ml) to preclude the interaction of ligand with the small amounts of Tac protein occasionally present on these cells. Scatchard analysis of the specific binding data revealed ~ 2,300 sites per cell assuming a uniform distribution of receptors within the cell population (Fig. 5). Also, a linear Scatchard plot suggested the presence of a single-affinity class of receptors having an apparent K_d of 200 pM. Similar binding assays performed in the absence of anti-Tac failed to reveal significant numbers of high affinity binding sites on these cells consistent with the lack of significant Tac antigen expression (data not shown).

As noted, IL-2 also stimulates a more delayed proliferative response within the NK cell population, which is blocked by the anti–Tac antibody. Thus, we next investigated the possibility that IL-2 stimulation, presumably acting through the p70 protein, modulated Tac gene expression. Cytoplasmic RNA was isolated from NK cells stimulated with 10 pM or 5 nM IL-2 for various times in the presence of a 200-fold molar excess of anti-Tac, blotted andd hybridized with a ^{32}P-labeled Tac cDNA fragment. As shown in Fig. 6 *A*, virtually no Tac mRNA expression was detected in freshly isolated NK cells or

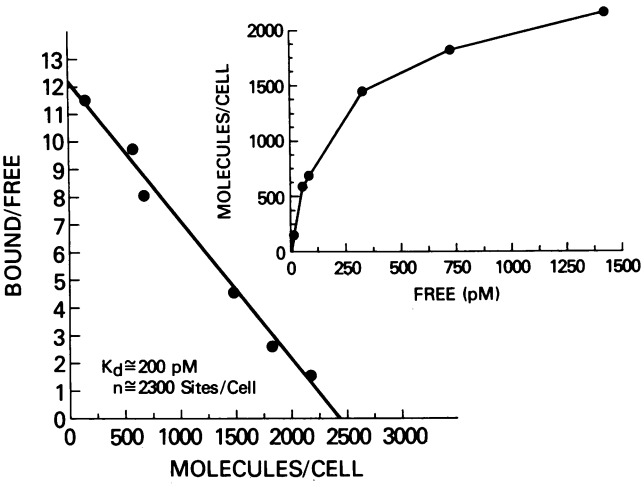

Figure 5. ^{125}I-IL-2 radioreceptor binding assays with NK cells. IL-2 binding sites on freshly isolated Leu 11$^+$ lymphocytes were analyzed by ^{125}I-IL-2 binding assays as detailed in the Methods. Scatchard analysis of the binding data is presented. All results are corrected for nonspecific binding as a ~ 35% loss of ligand binding activity incurred during the radiolabeling.

in cells activated with IL-2 for 2 h. In contrast, at 24 h, a marked increase in Tac mRNA was evident. Greater amounts of Tac mRNA were always present in the cell samples stimulated with 5 nM IL-2 compared with 10 pM IL-2, although a measurable response was consistently present at the lower IL-2 concentration. In experiments not shown, Tac mRNA was first detectable at ~ 12 h after IL-2 addition. Studies of c-myb mRNA expression revealed an early IL-2 induction of this gene occurring at 2 h. Also, these changes were sustained for at least 24 h. The rapid and prolonged induction of this gene raises the possibility that its gene product might play a role both in augmenting cytolytic activity and increasing cellular proliferation.

Discussion

In these studies, we have investigated the phenomenon of IL-2–induced activation of NK cells. In agreement with earlier studies, we have found that IL-2 enhancement of NK cytolytic function occurs rapidly even in the presence of the anti–Tac antibody, requires relatively high concentrations of IL-2 for optimal effects, and involves cells that largely or completely lack detectable expression of the Tac antigen. In contrast, IL-2–induced NK cell proliferation occurs with more delayed kinetics and is markedly inhibited by the anti–Tac antibody.

We have shown that freshly isolated NK cells contain on their cell surface a novel IL-2 binding protein. This IL-2 receptor is (*i*) constitutively expressed in the absence of cellular activation signals, (*ii*) ~ 70,000 D in size (p70) as assessed by the covalent cross-linking of ^{125}I-IL-2 , (*iii*) unreactive with the anti–Tac antibody, and (*iv*) apparently identical to the recently recognized second subunit of the human high affinity IL-2 receptor (12–16). Scatchard analyses of ^{125}I-IL-2 binding assays indicate that these highly enriched NK cells display ~ 2,300 p70 sites per cell that bind IL-2 with an apparent K_d of 200 pM. The constitutive expression of these intermediate affinity p70 binding sites suggests the possibility that these

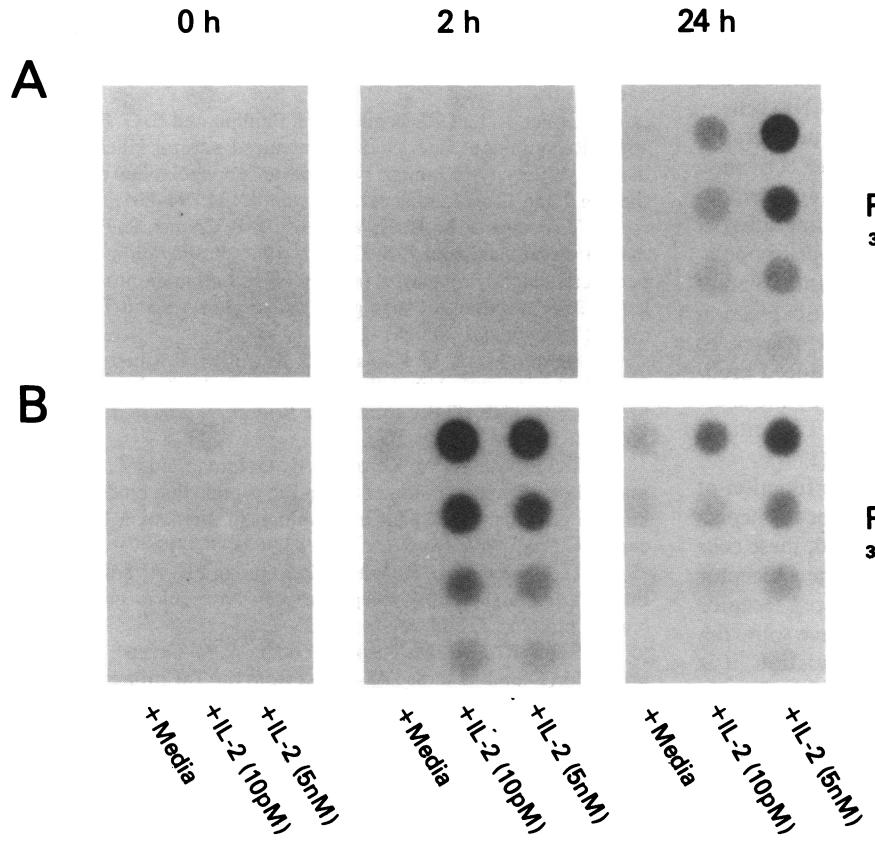

Figure 6. Induction of Tac and c-myb mRNA in IL-2–stimulated NK cells. Leu 11$^+$ cells incubated for varying time periods with IL-2 (5 nM or 10 pM) in the presence of a 200-fold molar excess of the anti–Tac antibody. Cells were harvested at the appropriate time, and cytoplasmic dot blots performed. Nitrocellulose membranes were hybridized either with a ^{32}P-labeled Tac cDNA fragment (*A*) or a c-myb cDNA fragment (*B*). 5 mM concentration of IL-2 is equivalent to 1,000 BRMP reference units per ml.

receptors may be importantly involved in transducing the requisite intracellular signals that lead to IL-2–induced enhancement of NK cytotoxicity. The inability of the anti–Tac antibody to block this action is also consistent with such a role for this receptor. Furthermore, the requirement for relatively high concentrations of IL-2 is not surprising given the intermediate affinity of these receptors for IL-2. However, formal proof that the p70 protein corresponds to the transducing structure in this response awaits the preparation of antireceptor antibodies that either block IL-2 binding or impair its capacity to function.

Our studies also demonstrate that high concentrations of IL-2 activate Tac gene expression in NK cells, probably via an interaction with the p70 protein. The subsequent production of Tac protein in these cells could then allow assembly of the high affinity IL-2 receptor complex that contains at least the Tac and p70 proteins. Such high affinity IL-2 receptors are clearly involved in IL-2–induced T cell proliferation, and, thus, by analogy, may play a similar role in IL-2–induced NK cell proliferation. The capacity of the anti–Tac antibody to inhibit IL-2–stimulated proliferation is consistent with this proposed formation of the high affinity IL-2 receptor complex.

Expression of the p70 IL-2 binding protein in the near or complete absence of the Tac antigen has also been detected on the "natural killer like" YT cell line (14, 15, 28). YT cells contain 8,000–10,000 p70 receptors that bind IL-2 with an apparent K_d of 810 pM. Activation of these cells with IL-2 or other stimuli, including forskolin, IL-1, or adult T cell leukemia factor (14, 15, 29, 30) also induces Tac gene expression and results in the expression of high affinity IL-2 receptors. Similarly, transfection of these cells with a plasmid containing of 5′ regulatory region of the Tac gene linked to the chloramphenicolacetyl transferase gene and subsequent stimulation with high concentrations of IL-2 leads to the activation of the Tac promoter (Taylor, A., and W. C. Greene, unpublished observations). Thus, IL-2 stimulation of this NK-like cell line recapitulates effects observed with freshly isolated NK cells.

High cconcentrations of IL-2 also produce a proliferative response in resting T cells in the absence of other activation signals (31, 32). Like NK cells, recent studies have demonstrated that resting T cells express ~ 600 p70 binding sites per cell (assuming a uniform distribution within the cell population) with an apparent K_d of 340 pM (17). IL-2 stimulation of these cells also induces c-myb, c-myc, and Tac gene expression that is not blocked by the anti–Tac antibody. However, as found with the NK cells, the subsequent IL-2–induced proliferative response in these cells is largely inhibited by the anti–Tac antibody, implying the requirement for the Tac antigen and the formation of high affinity IL-2 receptors (17).

In contrast to cytotoxic T cells, the cytotoxic function of NK cells does not require the recognition of major histocompatibility antigens nor is it antigen specific. Indeed, these cells often fail to express the T3 component of antigen receptor complex and often lack rearrangement within T cell receptor beta-chain gene locus (33). Whereas these cells have some degree of cytotoxic activity in the absence of stimulation, it is clear that this activity can be significantly enhanced by T cell–derived lymphokines produced in the course of antigen or mitogen stimulation. The relatively high constitutive expression of the p70 IL-2 binding protein on these cells certainly provides a plausible mechanism to explain the capacity of IL-2 to enhance the function of these NK cells.

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