Signal Transduction Mechanisms in Human Natural Killer Cells Mediating Antitumor Immunity

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A. Introduction

The spontaneous cytotoxicity of normal human peripheral blood mononuclear cells (PBMCs) is mediated by a subset of lymphocytes called natural killer (NK) cells. Unlike other cytotoxic lymphocytes, which require prior sensitization by the specific antigen to which they are programmed to respond, single NK cells are directly cytotoxic to a wide variety of malignant cells without prior exposure. Neither the NK cell receptor responsible for activation during this interaction nor its target cell cognate have been identified biochemically. NK cells can also be activated for lysis by the Fc region of IgG antibodies which have coated a malignant target cell (antibody-dependent cellular cytotoxicity, ADCC). In contrast to the situation in direct cytotoxicity, the NK cell surface Fc receptor (CD16) has been well characterized and can be identified by monoclonal antibodies (mAbs) such as 3G8 and Leu-11.

In this study, we used cloned, human NK cell lines to characterize the intracellular signal transduction pathways that are used during NK cell activation. NK cells activated by direct binding of sensitive tumor cells or by Fc receptor ligation by anti-CD16 antibody demonstrated a rapid increase in phosphoinositide hydrolysis. Moreover, this response was modulated in a heterologous manner by the cAMP second messenger pathway, a system known to exert a significant regulatory action on NK cell cytolytic activity.

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B. Materials and Methods

I. Isolation, Passage, and Characterization of NK Cell Lines

Adherent cell-depleted human PMBCs were stained with fluoresceinated anti-Leu-11a (CD16) and sorted on a FACS IV cell sorter. Using a modification of the procedure by van de Griend et al. [1], the separated populations (>98% $CD16^+$ upon reanalysis) were plated in limiting dilution (0.3–3.0 cells/well) with irradiated (4000 R) autologous PBMCs, irradiated (10000 R) allogeneic EBV-transformed cells, human recombinant IL-2 (20 units/ml), and 20% human sera. After 7 days, fresh IL-2 (10 units/ml) was added. On day 13, the replicate wells were scored for cell proliferation and clonal cell lines were selected based on the Poisson distribution. The cell lines were passaged weekly and their phenotype was monitored using fluorescent antibodies and flow cytometry.

II. Cytotoxicity Assay

The ^{51}Cr-release assay was performed using a procedure previously described [2]. Results are expressed as lytic units/10^6 cells, where 1 lytic unit is the number of cells required to give 20% specific chromium release [3]. Lytic units were calculated using computer software generously provided by Hugh F. Pross (Queens University, Kingston, Ontario).

III. Measurement of Inositol Phosphates

Inositol phosphate generation was evaluated using a modification [4] of the procedure previously described [5]. Brief-

ly, NK cells were prelabeled with myo[^{3}H]inositol and then incubated with either target cells (E/T ratio = 1/1) or monoclonal antibodies (1 μg/10^6 cells) in media containing 10 m*M* lithium chloride, an inhibitor of inositol-1-phosphatase. After various time intervals (1–60 min), the reactions were terminated by the addition of methanol:chloroform:HCl (200:100:2). After the addition of chloroform and water, the aqueous phase was collected, added to a 60 m*M* sodium formate-5 m*M* sodium tetraborate buffer, and loaded onto AG1-X8 anion-exchange columns. IP1 and IP2 were eluted from the column with 0.5 *M* ammonium formate – 100 m*M* formic acid. The remaining IP3 or, alternatively, the total inositol phosphates were eluted with 1.2 m*M* ammonium formate – 100 m*M* formic acid. [^{3}H]-labeled inositol phosphates were quantitated by liquid scintillation counting.

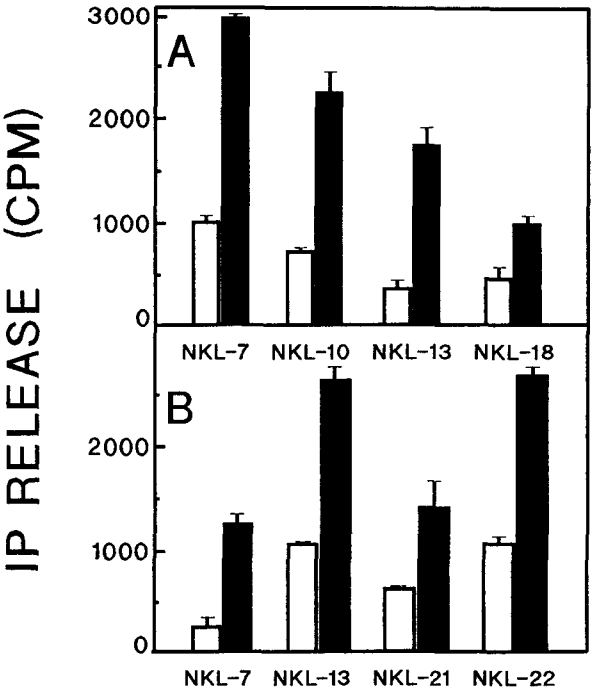

Fig. 1 A, B. Inositol phosphates are generated during binding of NK-sensitive targets (**A**) or during Fc receptor ligation (**B**). Different human CD16$^+$/CD3$^-$ NK cell lines were prelabeled with [^{3}H]myoinositol and incubated for 30 min with medium alone (*open bars* in **A**), K562 leukemic cells (*solid bars* in **A**), control antibody MKD6 (*open bars* in **B**), or with anti-CD16 antibody 3G8 (*solid bars* in **B**). Inositol phosphates were then extracted, partitioned by anion-exchange chromatography, and quantitated by liquid scintillation counting

C. Results and Discussion

In order specifically to assess signal transduction in homogeneous populations of human NK cells, cloned CD16$^+$/CD3$^-$ cell lines were selected and characterized. When these NK cell lines were exposed to NK-sensitive tumor targets for 30 min, the level of inositol phosphates rose two to five times above background (Fig. 1 A). To determine whether the target cell-induced inositol phosphate generation correlated with sensitivity to NK cell-mediated lysis, a panel of tumor target cell lines were comparatively evaluated in both inositol phosphate release and cytotoxicity assays (Fig. 2). There was complete concordance between target cell sensitivity to lysis and stimulation of phosphoinositide hydrolysis.

Since ADCC is generated by the crosslinking of Fc receptors on the NK cell surface, we also evaluated the direct effect of Fc receptor ligation using anti-Fc receptor antibodies (3G8). Incubation of the [^{3}H]inositol-labeled NK cell lines with 3G8 mAb consistently increased total inositol phosphate levels two to five times over those present in unstimulated cells (Fig. 1 B). Kinetics experiments demonstrated that stimulation by either susceptible target cells or by Fc receptor ligation led to rapid (1 min) generation of the Ca^{2+}-mobilizing second messenger inositol trisphosphate (IP3), with slower accumulation of inositol bisphosphate (IP2) and inositol monophosphate (IP1).

Previous studies have demonstrated that activation of the cyclic AMP-dependent second messenger pathway strongly inhibits NK cell-mediated cytotoxic functions. Treatment of NK effector cells with forskolin to elevate intracellular cAMP levels resulted in a concentration-dependent inhibition of phosphoinositide hydrolysis induced by both NK-sen-

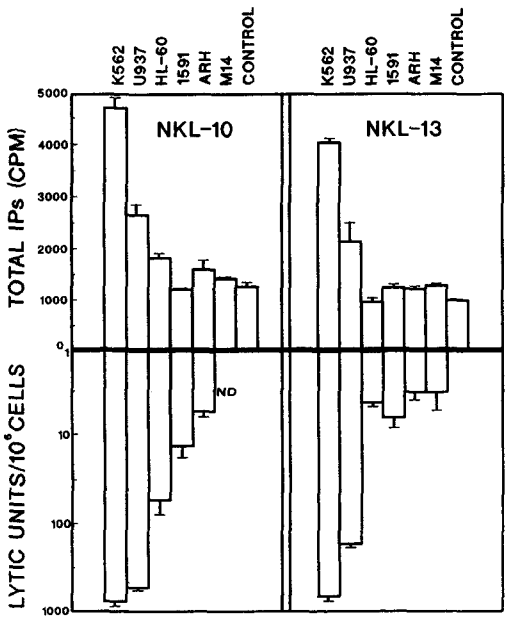

Fig. 2. Phosphoinositide metabolism is differentially stimulated by targets of varying sensitivity to NK lysis. Two human $CD16^+/CD3^-$ NK cell lines were incubated for 30 min with medium alone or with six different tumor targets. In parallel, the sensitivity of each tumor to NK cell-mediated lysis was measured in a 4-h ^{51}Cr-release assay

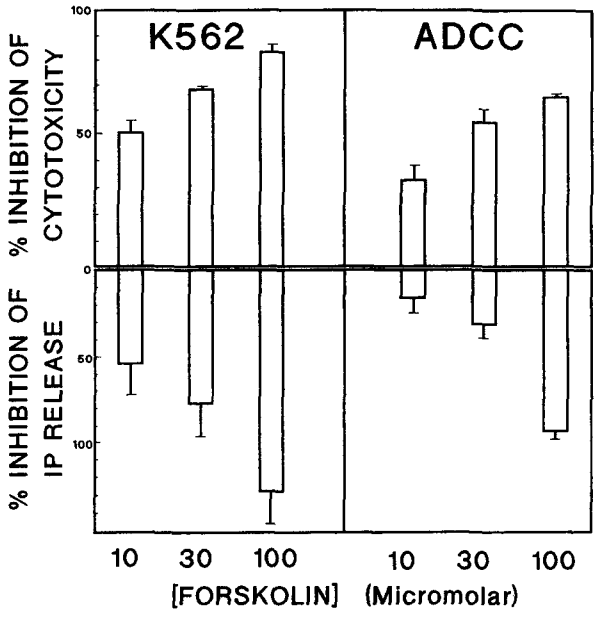

Fig. 3. Coordinate inhibition of NK cytotoxicity and inositol phosphate formation by intracellular cAMP elevations. The human NK cell line NKL-22 was preincubated for 10 min with various concentrations of forskolin and then assayed for direct and antibody-mediated cytotoxicity (*upper panels*). In parallel, forskolin-treated NK cells were tested for inositol phosphate release after stimulation by K562 cells or after Fc receptor ligation by antibody 3G8 (*lower panels*)

sitive targets and 3G8-mediated Fc receptor ligation (Fig. 3).

These results suggest that phosphoinositide turnover represents a critical early event in the human NK cell cytolytic process. Moreover, the potent inhibitory effect of cAMP on NK cell cytotoxicity may be explained by the uncoupling of NK receptors from phospholipase C-mediated phosphoinositide hydrolysis.

References

1. Van de Griend RJ, Van Krimpen BA, Bol SJ, Thompson A, Bolhuis RL (1984) Rapid expansion of human cytotoxic T cell clones: growth promotion by a heat-labile serum component and by various types of feeder cells. J Immunol Methods 56:285–298
2. Leibson PJ, Hunter-Laszlo M, Douvas GS, Hayward AR (1986) Impaired neonatal natural killer cell activity to herpes simplex virus: decreased inhibition of viral replication and altered response to lymphokines. J Clin Immunol 6:216–224
3. Pross HF, Bain MG, Rubin P, Sharagee P, Patterson MS (1981) Spontaneous human lymphocyte-mediated cytotoxicity against tumor target cells. IX. The quantitation of natural killer cell activity. J Clin Immunol 1:51
4. Abraham RT, Ho SN, Barna TJ, McKean DJ (1987) Transmembrane signaling during interleukin-1 dependent T cell activation. J Biol Chem 262:2719
5. Bijsterbasch MK, Meade CJ, Turner GA, Klaus GG (1985) B lymphocyte receptors and phosphoinositide degradation. Cell 41:999