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Research Article

Natural killer (NK) cells, which represent a small fraction of normal peripheral blood mononuclear cells, were purified by immunofluorescent cell sorting of NKH1+ cells. cytotoxicity of NKH1+ cells could be enhanced through activation by monoclonal antibodies (anti-T11(2) and anti-T11(3)) specific for epitopes of the sheep erythrocyte receptor or by recombinant interleukin-2 (rIL-2). After 18 h, incubation with both anti-T11(2/3) and rIL-2 resulted in similar levels of enhanced cytotoxicity against NK-resistant as well as NK-sensitive targets. Before and after induction, cytotoxicity was found predominantly within the NKH1+ population. These results suggest that several distinct mechanisms may be capable of enhancing NK activity and that the cells responsible for lymphokine-activated killing are likely to be the same population capable of spontaneous or natural killing before activation in vitro.

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Enhancement of Natural Killer Function Through Activation of the T11 E Rosette Receptor

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Abstract

Natural killer (NK) cells, which represent a small fraction of normal peripheral blood mononuclear cells, were purified by immunofluorescent cell sorting of NKH1⁺ cells. Cytotoxicity of NKH1⁺ cells could be enhanced through activation by monoclonal antibodies (anti-T11₂ and anti-T11₃) specific for epitopes of the sheep erythrocyte receptor or by recombinant interleukin-2 (rIL-2). After 18 h, incubation with both anti-T11_{2/3} and rIL-2 resulted in similar levels of enhanced cytotoxicity against NK-resistant as well as NK-sensitive targets. Before and after induction, cytotoxicity was found predominantly within the NKH1⁺ population. These results suggest that several distinct mechanisms may be capable of enhancing NK activity and that the cells responsible for lymphokine-activated killing are likely to be the same population capable of spontaneous or natural killing before activation *in vitro*.

Introduction

Natural killer (NK)¹ cells have been operationally defined as a population of lymphocytes capable of mediating direct cytotoxicity against a variety of target cells without prior immunization (1). Although NK cells in peripheral blood have been shown to be phenotypically and functionally heterogeneous (2), they appear morphologically as a relatively homogeneous population of large granular lymphocytes. Moreover, there are some NK-associated antigens that appear to be expressed on all NK cells and only rarely on other cell types. One of these antigens, termed NKH1, is a 200-Kd structure that is only expressed on 10–12% of peripheral blood mononuclear cells (PBMC) (3). NKH1⁺ cells appear morphologically as LGL. All NK activity in peripheral

blood is contained within the NKH1⁺ population. NKH1 is not expressed by B cells, monocytes, or granulocytes and is expressed only on a minor population of T cells. Of note, NKH1⁺T3⁺ cells in peripheral blood have also been shown to have broad cytolytic activity similar to that of NKH1⁺T3⁻ cells, which represent the majority of NK active cells (4).

Another antigen expressed on >80% of NK cells is the T11/E rosette receptor (CD2). The T11 antigen, which is expressed on all T cells and thymocytes, has been shown to be a pathway for antigen-independent activation of peripheral blood T lymphocytes (5). Three different epitopes on the T11 antigen have been defined. The T11₁ epitope is closely associated with the sheep red blood cell receptor and is expressed on thymocytes, T cells, and NK cells (3). The T11₂ epitope has the same tissue distribution as T11₁ but is spatially distinct from the receptor for SRBC. The T11₃ epitope is absent on resting T11⁺ cells but is induced following T cell activation (5). By using a combination of both anti-T11₂ and anti-T11₃ monoclonal antibodies, it is possible to activate T11⁺ cells *in vitro* and induce expression of surface receptors for IL-2. Activation through the T11 structure is independent of expression of T cell receptor for antigen and has been found to occur with immature thymocytes (6) as well as T3-negative NK clones (7). In addition, it has been demonstrated that cellular activation with anti-T11 monoclonal antibodies induces unrestricted killing by cytolytic T cell clones as well as cytotoxicity of NK clones against otherwise resistant targets (8). In the present studies, we demonstrate that the cytolytic function of peripheral blood NK cells can be markedly enhanced through activation of the CD2 and that this enhancement is comparable to that observed following IL-2 activation (9).

Methods

Monoclonal antibodies. The antibodies used in these studies have been previously described in detail. Anti-NKH1_A is an IgM monoclonal antibody with identical specificity to anti-NKH1 (N901) (3). Anti-T11₂ and anti-T11₃ define two different epitopes of the E rosette receptor (5). 2F12 is a monoclonal antibody recognizing the alpha chain of LFA-1 (10).

Cell lines. Molt4 is a T cell leukemic line. K562 is a cell line established from a patient with myelogenous leukemia. OH-1 is a small lung cell carcinoma line provided by Dr. S. Bernal (11). SKRC is derived from a renal carcinoma and referred elsewhere as SKRC-1 (12). Mewo is a melanoma cell line (13).

Analysis and purification of subpopulations of human PBMC. PBMC were isolated from healthy volunteer donors by Ficoll-Hypaque density gradient centrifugation. Nonadherent cells were obtained from PBMC by 1 h adherence on plastic culture dishes at 37°C. They were incubated with anti-NKH1_A for 30 min at 4°C, washed twice, and stained with fluorescein-conjugated goat anti-murine Ig for 30 min at 4°C. After two

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1. *Abbreviations used in this paper:* CD2, T11/E rosette receptor; E/T, effector/target ratio; LAK, lymphokine-activated killing; NK, natural killer; PBL, peripheral blood lymphocytes; PBMC, peripheral blood mononuclear cells; rIL-2, recombinant interleukin-2.

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additional washes, NKH1⁺ fluorescent cells were separated from non-fluorescent cells (NKH1⁻) using flow cytometric cell sorting (FACS I, Becton-Dickinson & Co., Mountain View, CA). Background fluorescence was determined with a nonreactive IgM antibody and positive cells were sorted in RPMI 1640 plus 1% pooled human AB serum at a rate of 3,000 cells/s. Reanalysis of purified populations confirmed that the positive fraction contained >90% fluorescent cells and the negative fraction contained <2% fluorescent cells.

Cytotoxicity assays. Cytotoxicity assays were performed according to a standard ⁵¹Cr release method previously described (3). After cell sorter purification, fractionated cells were incubated with either recombinant interleukin-2 (rIL-2) medium (1,000 U/ml; Biogen S. A., Geneva, Switzerland) or anti-T11₂ and anti-T11₃ media (1/250 final dilution of ascites) in microtiter plates for 18 h at 37°C before adding ⁵¹Cr-labeled target cells. All reagents were subsequently left in the wells throughout the 4-h cytotoxicity assay. 18-h incubation with anti-T11 antibodies was found to be optimal for induction of cytotoxicity. Longer incubations with these reagents resulted in less enhancement. In contrast, rIL-2-induced cytotoxicity was clearly evident at 18 h but in some instances was not maximal until after 48 h of incubation. All experiments were done in triplicate using V bottom microtiter plates. Medium was RPMI 1640 plus 5% pooled human AB serum containing 50 U/ml penicillin and 50 µg/ml streptomycin. Assays were performed at various effector/target (E/T) ratios using between 3,000 and 5,000 targets cells per well. With all targets used, the minimum release (targets incubated in media alone) was <20% of the maximum release (targets incubated in 1% Triton-X detergent). The results shown are the mean of triplicate experiments. Standard deviation is always <5%.

Conjugate formation assay. NKH1⁺ and NKH1⁻ sorted populations were incubated with either anti-T11₂ and anti-T11₃ monoclonal antibody media (1:250 final ascites dilution) or rIL-2 medium (1,000 U/ml) for 18 h at 37°C. In control experiments, cells were incubated with anti-T11₃ and anti-T1, another monoclonal antibody of the same isotype as anti-T11₂. Following incubation, cells were mixed with K562 targets at an E/T ratio of 20:1 in a final volume of 0.5 ml and centrifuged for 5 min at 800 rpm. After various further incubation periods at 37°C, the cells were gently resuspended with a pasteur pipette. Percent target cells forming conjugates with at least one effector cell was determined after enumeration of 200 K562 cells.

Results

Induction of cytotoxic activity in NKH1⁺ cells. Human NK cells were purified from peripheral blood by immunofluorescent-flow cytometric cell sorting of NKH1⁺ cells. When PBMC are separated into NKH1⁺ and NKH1⁻ fractions (Fig. 1 A) all of the natural cytotoxicity against a standard NK target cell, K562, is contained within the NKH1⁺ population. After 18-h incubation with rIL-2 (Fig. 1 B), the NK activity of unseparated PBMC is significantly enhanced and cytotoxicity remains confined to the NKH1⁺ subset. Results shown in Fig. 1 C demonstrate that en-

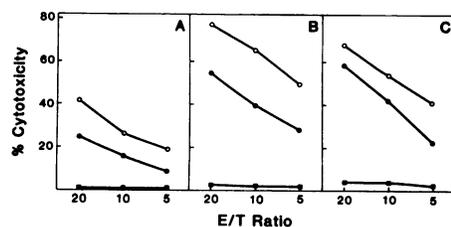


Figure 1. Activation of cytotoxicity against K562. NK activity of PBL (solid circle), NKH1⁺ (open circle), and NKH1⁻ (solid box) cells was measured against K562 target cells after incubation with either media (A), purified IL-2 (B), or with anti-T11_{2/3} monoclonal antibodies (C).

hancement of cytotoxicity can also be seen following 18-h incubation with anti-T11_{2/3} monoclonal antibodies. Moreover, the triggering of peripheral blood NK cells with anti-T11_{2/3} antibodies is as effective as with rIL-2 and is also restricted to NKH1⁺ cells.

Induction of conjugate formation following NK cell activation.

To directly examine the effect of anti-T11 monoclonal antibodies on effector-target cell binding, we evaluated the ability of activated effector cells to form conjugates with K562 targets. As shown in Table I, preincubation of NKH1⁺ cells with either anti-T11_{2/3} or rIL-2 significantly enhances conjugate formation. This effect is stronger following anti-T11_{2/3} than following rIL-2 activation. Interestingly, conjugate formation is also induced in NKH1⁻ cells when activated through the T11 pathway although these cells are not able to mediate direct cytotoxicity. In contrast, rIL-2 does not induce the formation of conjugates in the NKH1⁻ population. In control experiments with the combination of monoclonal anti-T1 and anti-T11₃, there was no induction of cytotoxicity or conjugate formation with K562 targets (data not shown).

Effect of anti-LFA-1 antibody on induced cytotoxicity. The LFA-1 antigen has been shown previously to play an important role in the cell-cell interactions and formation of conjugates that occur during the cytolytic process of NK and other cytolytic cells (14). To characterize the role of LFA-1-mediated cell adhesion during T11-induced cytotoxicity, we added monoclonal 2F12 antibody to cells during activation before cytotoxicity assays. As shown in Fig. 2 B, T11-mediated enhancement of cytotoxicity can be induced against additional NK target cells such as Molt4 resulting in more than a two-fold increase in cytotoxic activity after 18 h incubation at 37°C. As seen previously with other target cells, cytotoxicity is contained within the NKH1⁺ fraction; very little killing can be found in the NKH1⁻ fraction either before or after T11 activation. When 2F12 monoclonal antibody is added to the NK assay (Fig. 2 D), cytotoxicity of T11-induced cells is reduced by >50% and is similar to that observed before activation. These results suggest that the T11-induced enhancement of cytotoxicity is at least in part due to an increased binding of effectors to target cells, mediated through LFA-1 antigen. Similar results were obtained with 2F12 antibody and rIL-2-activated NK cells (data not shown).

Induction of cytotoxicity against resistant targets. To test whether T11 activation would induce cytotoxicity against NK-resistant tumor cells, we performed similar experiments with

Table I. Effector-Target Cell Conjugates Induced by Anti-T11_{2/3} and rIL-2

Time	NKH1 ⁺ cells			NKH1 ⁻ cells		
	Media	Anti-T11 _{2/3}	rIL-2	Media	Anti-T11 _{2/3}	rIL-2
20'	10*	50	44	4	88	0
60'	10	68	52	8	94	8
120'	32	72	46	6	96	2

Peripheral blood mononuclear cells were separated into NKH1⁺ and NKH1⁻ populations by immunofluorescent cell sorting and subsequently incubated with either anti-T11₂ and anti-T11₃ or rIL-2. Following 18 h incubation, effector cells were incubated with K562 target cells for 20, 60 or 120 min at 37°C before enumeration of conjugates. * Each value represents percent K562 target cells forming conjugates with at least one effector cell.

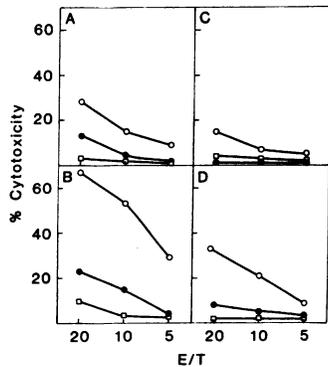


Figure 2. Effect of anti-LFA-1 antibody on cytotoxicity following T11 activation. Cytotoxic activity of PBL (solid circle), NKH1⁺ (open circle), and NKH1⁻ (open box) effector cells against Molt4 target cells following incubation with media (A), anti-T11_{2/3} monoclonal antibodies (B), anti-LFA-1 monoclonal antibody at 1/250 final dilution of ascites (C), or anti-LFA-1 and anti-T11_{2/3} antibodies (D).

three different solid tumor cell lines as targets. As shown in Fig. 3, the melanoma cell line Mewo and the renal cell carcinoma line SKRC are not killed by peripheral blood lymphocytes (PBL) incubated in media only. PBL are able to exhibit minimal killing (7%) against the small-cell lung carcinoma line OH-1. After activation by anti-T11_{2/3}, PBL exert significantly more cytotoxicity against each of the three lines (14%, 14%, and 17%, respectively). Comparing the cytotoxic activity of NKH1⁺ and NKH1⁻ sorted cells after incubation in media only, we observed that NKH1⁺ cells are able to mediate a low level of killing against OH-1 cells (14% cytotoxicity) but very little cytotoxicity (<6%) against Mewo and SKRC targets. NKH1⁻ cells are not able to kill any of these targets. After T11_{2/3} activation, cytotoxicity of NKH1⁺ cells is enhanced against all three targets, but NKH1⁻ effectors have very little activity (always <6%). Similar induction of cytotoxicity within the NKH1⁺ population was observed following activation of effector cells with rIL-2. This data therefore supports the hypothesis that NKH1⁺ cells are responsible for both rIL-2 and T11-induced killing of NK-resistant targets by PBL.

Discussion

In summary, this study demonstrates that the cytotoxic function of purified NKH1⁺ peripheral blood NK cells can be significantly enhanced via the CD2 pathway. The extent of NK enhancement after an 18-h incubation period is quite similar to the effects seen after rIL-2 activation. Moreover, our data indicate that activation with either rIL-2 or anti-T11_{2/3} monoclonal antibodies induces cytotoxicity against otherwise resistant target cells. The cell separation studies done in conjunction with in vitro acti-

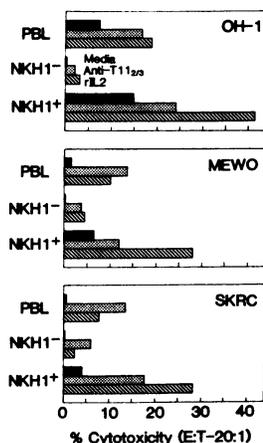


Figure 3. Induction of cytotoxicity against NK-resistant targets. Assays were performed with PBL or purified NKH1⁺ and NKH1⁻ fractions after 18 h incubation of the effectors with either media (solid bar), anti-T11_{2/3} antibodies (stippled bar), and rIL-2 (hatched bar). OH-1 is a small-cell lung carcinoma line, Mewo is a melanoma line, and SKRC is the renal carcinoma cell line (SKRC-1).

vation consistently demonstrate that cytotoxicity is contained almost exclusively within the NKH1⁺ cell fraction.

Although IL-2 and T11 activation lead to similar degrees of enhancement of cytotoxicity, there are differences in the effects of rIL-2 and anti-T11_{2/3} that suggest that distinct cellular mechanisms may be involved. One major difference is the enhancement of conjugate formation in the NKH1⁻ population that can be induced by anti-T11_{2/3} but not rIL-2 (Table I). It is also known that 1 h incubation with IL-2 is sufficient for enhancement of cytotoxicity (9, 15) whereas the effects of anti-T11 antibodies require at least 6–8 h of stimulation before significant enhancement can be detected. Longer periods of incubation (48–72 h) result in decreased cytotoxicity (data not shown). The effects of these two activators on cloned NK and cytotoxic T lymphocyte effectors have also shown different results because only anti-T11_{2/3} is able to induce killing of resistant targets by cultured cell lines (8). In addition we have observed that rIL-2 induces proliferation as well as cytotoxicity of NKH1⁺-purified cells whereas anti-T11_{2/3} activation does not induce in vitro proliferation of these cells (data not shown and 16). Nevertheless it is known that T11_{2/3} activation results in rapid expression of IL-2 receptor, and it is therefore possible that IL-2 may also play a significant role in the functional effects seen after stimulation with anti-T11_{2/3}. In this regard, the addition of both IL-2 and anti-T11_{2/3} antibodies does not further enhance the activation of NKH1⁺ cells seen after either stimulus alone (data not shown). Further studies will be necessary to explore the different roles and potential mechanisms of interaction between IL-2 and T11 activation in the regulation of NK activity.

Another important issue addressed by the present studies is the identification of those cells capable of mediating cytotoxicity after in vitro activation. Other investigators have previously demonstrated that a subset of cells in normal peripheral blood can be induced to kill spontaneously a variety of target cells after in vitro incubation with rIL-2 (17). Clinical studies utilizing in vitro activated cytotoxic cells in patients with metastatic cancer have been reported recently (18). These cells have been termed lymphokine activated killer (LAK) cells, and some studies have suggested that they are distinct from both T cells and NK cells (19). The results presented in this report consistently demonstrate that spontaneous cytotoxicity against a variety of target cells is exclusively contained within the NKH1⁺ population of PBL. In vitro activation by either rIL-2 or anti-T11_{2/3} results in marked enhancement of cytotoxicity against NK-sensitive targets as well as simultaneous induction of cytotoxicity against previously NK-resistant targets. However with both stimulated and unstimulated effector cells, enhanced cytotoxicity remains confined to the NKH1⁺ population that only represents a small fraction (~12%) of PBL. These studies therefore suggest that lymphokine-activated killing is a direct result of the activation of NKH1⁺ NK cells and argues against the presence of an additional population of cytolytic cells with a broad spectrum of reactivity. Similar findings have recently been reported by Lanier et al. (20). Taken together, these studies support the view that the LAK phenomenon primarily reflects the functional effects of various lymphokines in the regulation of NK activity in vivo.

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