EFFECTOR:TARGET INTERACTIONS IN THE HUMAN NATURAL KILLER CELL SYSTEM

CHARACTERIZATION OF THE TARGET STRUCTURES

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MATERIALS & METHODS

RESULTS

DISCUSSION

The three papers that follow represent the Materials and Methods, Results and Discussion sections of this dissertation. These papers are reprints of articles published in the established literature and subsequently will be referred to by the following labels:

Publication 1: Target Cell Specificity of Human Natural Killer (NK) cells I. Development of an NK-Resistant Subline of K562

Stephen L. MacDougall, Chaim Shustik and Arthur K. Sullivan

Cellular Immunology 76: 39-48 (1983).

Publication 2: Target Cell Specificity of Human Natural Killer (NK) Cells II. Apparent Change with Activation

Stephen L. MacDougall, Chaim Shustik and Arthur K. Sullivan

Cellular Immunology 103: 352-364 (1986).

Publication 3: Increased fucosylation of glycolipids in a human leukemia cell line (K562-Clone I) with decreased sensitivity to NK-mediated lysis

S. L. MacDougall, G. A. Schwarting, D. Parkinson and A. K. Sullivan

Immunology 62: 75-80 (1987).

METHODS

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Abstract

Natural killers (NK) are a subpopulation of lymphocytes that are defined by their pattern of cytotoxicity against other normal and neoplastic cells of predominantly hemopoietic origin. Although they have been programmed to recognize a limited spectrum of targets, their activity can be augmented by certain immunoregulatory substances. The molecules that mediate the recognition events have not yet been identified.

I describe here the derivation and characteristics of a variant clone (Clone I) of the human leukemic cell line K562. These cells, selected for decreased binding to peripheral blood lymphocytes, were less sensitive than the parent to lysis by NK in the resting, but not in the augmented state. Although their major plasma membrane proteins appeared identical to those of K562, they contained an additional minor group of fucosylated glycolipids. A later subclone of Clone I, selected for resistance to Concanavalin A, reverted to an NK sensitive pattern and exhibited the parental profile of glycolipids.

These results illustrate in an in vitro model how a leukemic cell can modulate its membrane to escape surveillance by NK cells, and suggest that the glycolipids might be involved (directly or indirectly) in the mechanism.

Résumé

Les cellules tueuses naturelles (cellules NK) constituent une sous-population de lymphocytes qui exercent une activité lytique sur certaines cellules normales ou néoplasiques. L'activité lytique des cellules NK peut être stimulée par différentes substances immunorégulatrices, même si l'on ignore quelles sont les molécules qui déterminent la sensibilité des cellules aux lymphocytes NK, qu'ils soient au repos ou soumis à une stimulation. Les études dont nous rendons compte décrivent la dérivation et la caractérisation d'une variante (Clone I) de la cible habituelle des lymphocytes NK (la K562) qui leur résiste. Le Clone I résiste spécifiquement aux cellules NK qui n'ont pas été stimulées et semble afficher une réduction, sinon une absence ou une inhibition des structures que visent les cellules NK. La comparison des membranes plasmatiques de Clone I et de K562 n'a fait ressortir aucune différence au niveau des principales protéines de surface: toutefois, on a observé sur le Clone I un groupe mineur de glycolipides membranaires fucosylés qui n'etaient pas présents sur K562. Un sous-clone de Clone I, résistant au Con A, est redevenu sensible aux cellules NK et a retrouvé le profil des glycolipides de la cellule mère. Ces résultats portent à croire que la résistance à la lyse affichée par les cellules NK serait le fait d'une modulation de la glycosylation des lipides de la membrane et témoignait de l'existence d'un mécanisme par lequel une cellule tumorale peut échapper à la vigilance immune des cellules tueuses naturelles. La sensibilité différentielle de ces souches cellulaires aux lymphocytes NK, qu'ils soient au repos ou soumis à une stimulation, en fait des intruments précieux dans l'étude de la spécificité des cibles de ces cellules effectrices.

Thesis Format Statement

This dissertation contains duplicated published text of original papers (**Publication 1, Publication 2,** and **Publication 3**) in the **MATERIALS & METHODS, RESULTS, DISCUSSION** section). In accordance with the "Guidelines Concerning Thesis Preparation", Faculty of Graduate Studies and Research, the text of section 7 of the Guidelines is hereby cited in full:

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The candidate has the option, subject to the approval of the Department, of including as part of the thesis the text, or duplicated published text (see below), of an original paper, or papers. In this case the thesis must still conform to all other requirements explained in Guidelines Concerning Thesis Preparation. Additional material (procedural and design data as well as descriptions of equipment) must be provided in sufficient detail (e.g. in appendices) to allow a clear and precise judgement to be made of the importance and originality of the research reported. The thesis should be more than a mere collection of manuscripts published or to be published. It must include a general abstract, a full introduction and literature review and a final overall conclusion. Connecting texts which provide logical bridges between different manuscripts are usually desirable in the interests of cohesion.

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THE TEXT OF THE ABOVE SHALL BE CITED IN FULL IN THE INTRODUCTORY SECTIONS OF ANY THESES TO WHICH IT APPLIES.

INTRODUCTION

It has been proposed that the immune system normally may be able to inhibit the progression of neoplastic cells by destroying them before they proliferate further and become clinically significant (25). This concept of immune surveillance against tumors initially was formulated with cytotoxic T-lymphocytes as the central mediator. These T-lymphocytes exhibit an extraordinary range of specificities, a result of recombination events in the T-cell receptor gene family (31). However, the T-cell receptor must interact with tumor-associated antigens presented in association with major histocompatibility molecules (225). The T-lymphocyte consequently must be activated and undergo a regulated clonal expansion to yield a functional population of cytotoxic effector cells. These requirements are critical in directing, controlling, and restricting the function of T-cell mediated immunity. Although these requirements do not preclude T-lymphocytes from playing a role in surveillance against spontaneous tumors, their response is adaptive and evidence indicates that T-cell mediated immunity is more efficient in the control of tumors induced by the oncogenic viruses and chemical carcinogens (98). The discovery of natural killer cells and the concept of natural immunity have led to the notion that such mechanisms might be mediators of immune surveillance against spontaneous tumors (72,73).

The phenomenon of natural killing initially was discovered during studies on cell-mediated cytotoxicity in tumor-bearing individuals (154,183). These individuals were

expected to exhibit specific cytotoxic activity against autologous tumor cells or against tumor cells of a similar type. However, control studies in healthy individuals revealed significant lymphocyte-mediated killing of some tumor cells and tumor cell lines. The effector cells mediating this cytotoxicity were given the term natural killer (NK) cells, and have been defined as a sub-population of lymphocytes with the potential to lyse spontaneously various types of target cells (72,73,154,189). The natural killer cell lacks the properties of classic macrophages, granulocytes, and cytotoxic T-lymphocytes, in that their activity does not require prior exposure to the target cell, and is not dependent on interaction with molecules of the major histocompatibility complex (72,73). Furthermore, natural killer cell activity can be significantly increased by a variety of immunoregulators (72,73). These characteristics have presented the natural killer cell as a possible first line of defence in the control of spontaneous tumor growth, particularly in the hematopoietic system.

Research over the past decade has produced much information about the morphology, lineage, mechanism of action, and regulation of the natural killer cell and has supported the hypothesis that natural killer cells are an important part of the immune network. However, several key questions remain to be answered, one of these being the nature of the target structures on the tumor cell membrane that are recognized by the natural killer cell.

The studies described in this dissertation attempt to define the plasma membrane components that are involved in determining target cell sensitivity to NK-mediated cytolysis, and to determine if the specificity of NK activity changes upon augmentation with immunoregulatory substances.

CHARACTERIZATION AND PROPERTIES OF NK CELLS

Morphology and Phenotype

Human natural killers initially were identified as nonadherent, nonphagocytic cells with lymphoid morphology (204). Saksela et al (170) extended this definition, using the technique of adsorption and elution of effector cells from targets. The eluted effectors were large lymphocytes with abundant cytoplasm containing numerous azurophilic granules. Almost all of the NK activity in the peripheral blood can be attributed to cells with large granular lymphocyte (LGL) morphology (186,187). However, some activated T-lymphocytes may also exhibit LGL morphology (55,103,104). The LGL population makes up approximately 5% of total peripheral blood lymphocytes (187); such cells are also found in spleen, bone marrow and lymph nodes (72,73,186,187). Natural killer cell activity correlates well with LGL frequency in these organs.

Despite the homogeneous morphology of LGL, characterization of their cell surface markers has revealed considerable heterogeneity. Most human NK activity (>90%) is mediated by cells bearing the $CD3^-$, $CD56^+$ ($NKH1^+$), $CD16^+$ (Fc gamma receptor) phenotype (138). Subsets can be derived according to the expression of a vast range of surface markers, some shared with cells of the myelomonocytic lineage (CD11b), some with cells of the T-cell lineage (CD2, CD7, CD8, CD25), as well as other markers (CD11a, CD57 or HNK-1) (73,137,138). None of these subsets has any exclusive functional correlate, and a cell surface marker unique to natural

killer cells has yet to be defined. The natural killer cell phenotype also can be defined by what cell surface markers are not expressed. Among these are most, if not all B-lymphocyte markers (CD20, CD21, surface Ig), as well as the T-cell markers CD4 and the monocyte specific CD14 and CD15 (73,137,138,189).

Independent of other evidence, this heterogeneity in the expression of cell surface markers implies that there may be some functional heterogeneity. This is supported by observations that different subsets of the $CD16^+$, $CD2^+$, $CD3^-$ phenotype produce different cytokines (138). Conversely, natural killer cell heterogeneity has made it difficult to definitively study NK function because the operating definition of the natural killer cell phenotype has varied arbitrarily among different investigators.

Lineage of NK Cells

The large granular lymphocytes that mediate natural killing share several morphologic and/or phenotypic features with both T-lymphocytes and monocytes. Because of this, various authors have assigned LGL to either the T-cell lineage, the myelomonocytic lineage, or to a separate lineage with a precursor common to both T-cells and monocytes. Alternatively, others have considered the NK population to be a mixture of cells derived from both T-cell and monocytic precursors.

It has been argued that LGL belong to the myelomonocytic lineage based on similarities in morphology, the presence of beta-glucuronidase and other enzymes in cytoplasmic granules (72,137,170,186), apparent marrow dependence (62), and expression of some common cell surface markers (e.g. CD11b) (9,93). However, this evidence is not conclusive because some of these shared characteristics are not specific to myelomonocytic lineage cells. When the expression of a more myeloid-specific marker was analysed (i.e. cell surface CSF-1 receptors), there appeared not to be phenotypic similarities between LGL and myelomonocytic cells (56).

Many other phenotypic and functional characteristics are shared by T lymphocytes and NK. Natural killer cells can form rosettes with sheep erythrocytes (140,204), express some T-cell associated markers (103,104,137,140), and are able to proliferate in the presence of interleukin-2 (42,102,138). As well, NK cells and cytotoxic T-lymphocytes appear to share a common lytic mechanism such as the active secretion of perforin-containing granules (217). However, mature T-cells mediate their effector function through the CD3 and T-cell receptor complex of

molecules and exhibit MHC restriction. This is not the case for NK cells, which express neither CD3, nor require T-cell receptor gene rearrangement for function (105,137,213). Studies of IL-2 dependent NK cell clones have revealed some $CD3^+$ cells with some rearrangement of T-cell receptor genes (103,138). These cells are, by definition, activated by IL-2 which may result in activation of their lytic potential in an antigen independent fashion. These cells appear with extremely low frequency, and exhibit other differences from typical peripheral blood NK cells (103,138). Therefore, it has been suggested that these clones represent activated T-cells and not NK cells.

Further divergence in NK and T-cell behavior is found in their pathway of differentiation. T-lymphocytes are typically thymus dependent (122,142). Reconstitution experiments in mice have shown a different pattern of emergence for T-cell and NK mediated immunity. NK function appears well before T-cell function. Athymic mouse strains often have normal NK characteristics and activity (75,96). In contrast, NK cells appear to originate and differentiate in the bone marrow (58,62,63,152). Experiments using lethally irradiated mice reconstituted with bone marrow have shown that NK activity (either high or low) depends on the phenotype of the donor marrow (18). Similar observations have been made in patients following therapeutic bone marrow transplantation. Both NK number and activity are initially depressed but are rapidly recovered after transplantation, unlike T-cell mediated function (8,13,41,168,189). The appearance of cells with LGL morphology closely follows the appearance of NK activity in these bone marrow recipients.

An hypothesis has been proposed suggesting that NK cells exhibit T-cell and myelomonocytic characteristics because they are a mixture of cells representing both

lineages (137,189). This hypothesis has not been disproven, and has not explained how cells from two distinct lineages can share LGL morphology, cytotoxic mechanisms, and other characteristics.

An alternative hypothesis has been presented by Grossman and Herberman (56). They suggest that the differentiation of NK and T-cells are intimately related, adaptive, and dependent on the nature of the self environment. Several key events in the pathway of differentiation would decide the rate of maturation, phenotypic plasticity, and subsequent functional activity. One of these events presumably would be rearrangement of the T-cell receptor genes. This model accounts for most of the shared and divergent characteristics of T-cells and NK cells. Grossman and Herberman stress that knowledge of the NK recognition structures is a central issue in defining NK cells and in furthering the development of their hypothesis (56).

Regulation of NK cells

The most potent immunoregulators of NK function are the interferons (73,76,187) and interleukin-2 (42,71,189), both of which augment NK cell activity Activation by interferons does not require the proliferation of NK cells (190), but does require de novo RNA (136) and protein synthesis (190). In mice, a subset of NK cells has been described that is not activated by interferon (123). A parallel observation has not been made in humans. Many other physiological substances are able to modulate NK function (72,73,86,189,and refs. within). These include beta-endorphins, arachidonic acid metabolites, corticosteroids, growth factors such as

platelet-derived growth factor, and a variety of other cytokines and hormones. Viral, bacterial and tumor cell products also have been shown to modulate NK activity.

The functional consequences of activation of NK by either interferon or interleukin-2 may include the following. 1) An increase in the proportion of LGL that bind to target cells (especially targets with low susceptibility to unactivated NK) (47,57,176,187). 2) An increase in the proportion of lytically active LGL (57,176,187). 3) An increase in the rate of killing by lytically active cells (47,176,187). 4) An increase in recycling of LGL (187,194), or at least an inhibition of the LGL inactivation process following effector-target interaction (1,145). 5) An increase in the range of susceptible target cells. 6) An increase in secretion of cytokines (137,138,and refs. within). Transient expression of the proto-oncogenes c-myb (100) and c-fos (32) has been observed in NK cells undergoing functional activation with IFN and IL-2. However, this enhanced expression may not be an absolute requirement for the activation process.

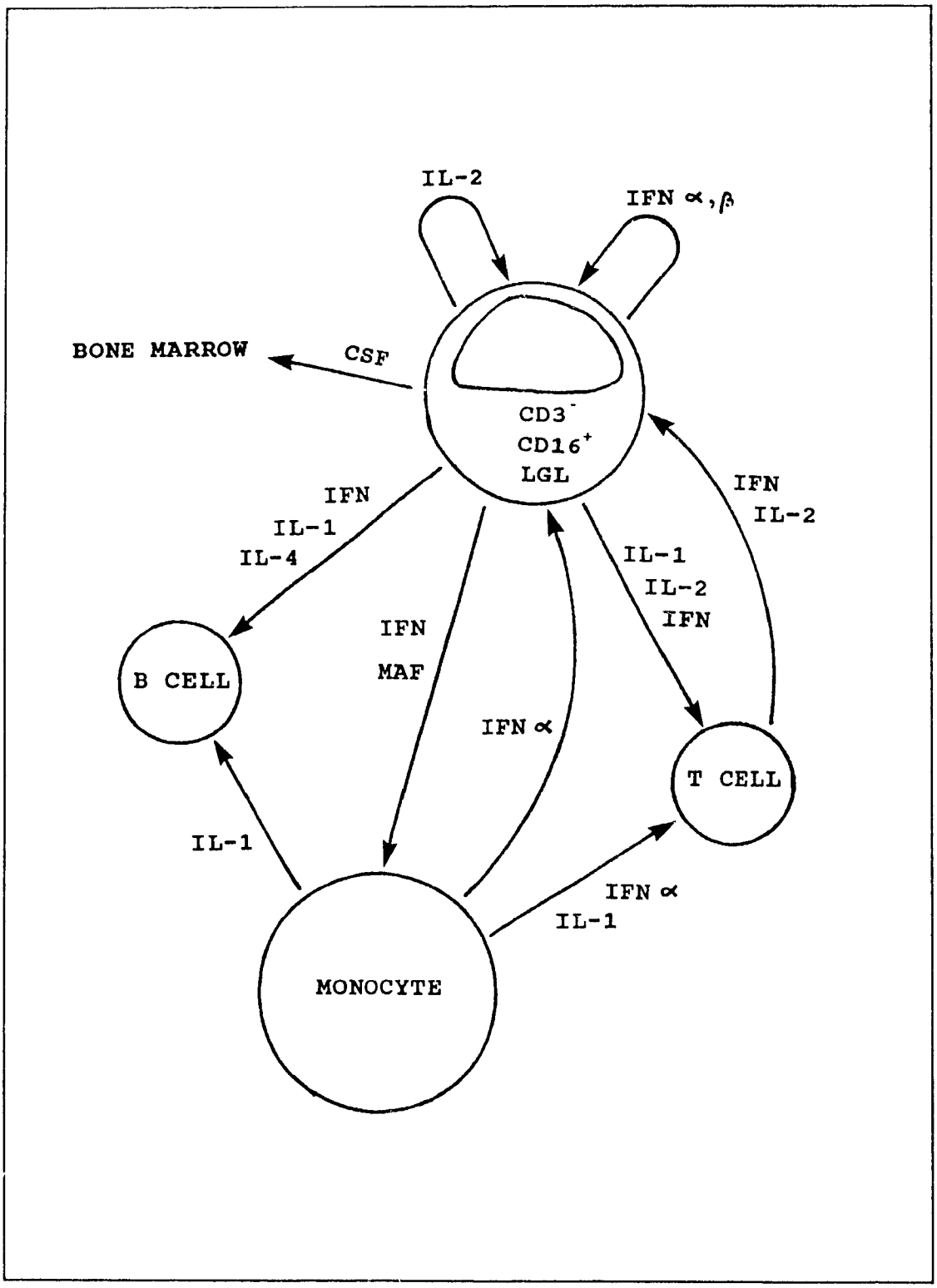
Activated NK cells are more efficient effectors, and may commur' 'ate more readily with other cells of the immune system. Responsiveness to interferon and IL-2 suggests that in conditions where interferon and/or IL-2 levels are elevated, activated NK cells are generated. Such conditions could include viral and bacterial infections, exposure of NK cells to tumor targets, or activation and proliferation of T-lymphocytes.

NK cells, as well as being targets for cytokines, are themselves able to secrete the IL-1, IL-2, IL-4, BCGF, BCDF, interferon alpha, beta and gamma, MAF and CSF (91,97,138,150,172,199). Studies using NK cell clones show that most NK are able to secrete at least interferon-gamma (137,138). Many of them secrete other

cytokines, with a few secreting multiple cytokines. This functional segregation is supported by studies using fresh unactivated NK cells (138).

All of the cytokines secreted by NK cells are able to modulate other immune cells, and this firmly establishes the NK cell as an important immune regulator. The fact that NK cells themselves can be targets for many of the same lymphokines suggests that self-regulation may play a central role in NK physiology. **Figure 1** illustrates some components of this regulatory network.

Figure 1. Lymphokine pathways in the immune system.



In vivo Functions of NK Cells

A large body of circumstantial evidence has been accumulated and suggests that natural killer cells can exert their cytotoxic activity against tumors in vivo. Although in vitro studies had shown that NK cells could effectively lyse tumor cells, the first clue that this system could function in vivo came when it was discovered that athymic, T-cell deficient mice could still eliminate tumors (96). These mice have increased levels of NK activity (202). Further experiments using animal models have shown that the metastatic capability of tumor cells correlates well with their resistance to NK-mediated lysis (49). Conversely, in strains of low-NK and high-NK mice, the development of metastases following a tumor challenge exhibits the expected pattern (160). Natural killer cell activity in mice can be significantly reduced by cyclophosphamide (158), treatment with ^{89}Sr (63), split dose irradiation (144), and anti-asialo G_{M1}antisera (92,174). In mice rendered NK-deficient by these means, there is an increase in tumor takes and metastatic spread following inoculation with tumor cells (50,92,158,174). In some cases, mice rendered NK deficient have been reconstituted with bone marrow (50), or the injection of purified NK cells or NK clones (201). These animals then are able to limit the development of primary tumors and metastatic spread as effectively as normal mice. In animals reconstituted with bone marrow, the ability to inhibit tumor growth is dependent on the NK activity of the donor strain (49). The beige mouse exhibits a genetically determined defect in NK function, and NK cells from these mice are not lytically active (164). There is a significant increase in the appearance of spontaneous

tumors in these mice, particularly tumors of lymphoid origin (60). As well, the ability of NK susceptible tumors to grow and metastasize is increased in beige mice (60,89,184). In general, increases in spontaneous tumors are seen in mouse strains with low NK activity. Together, these studies show that NK cells can be effective in vivo in the control of tumor growth in animals.

Evidence supporting the role of NK cells in tumor control in humans is not as extensive. Most of the data is correlational, a disease or condition correlating with an alteration in NK function and/or number. Conversely, an alteration in NK function and/or number may be associated with certain diseases or conditions. Natural killer cell activity is altered in a wide range of clinical conditions including infectious, endocrine, neurological, immunological, as well as other systemic conditions (see refs. 72, 73 for examples). The cause-effect relationships are not clear in any of these cases. As well, in many of these studies cytotoxicity against a limited number of cultured target lines has been used as the sole indicator of NK function. These limitations are apparent in studies of many forms of cancer where NK function often is depressed, such as some advanced solid tumors and leukemias (179,182,224 for example). The examination of cumulative data in cancer patients, careful investigation of NK function, and analysis of both tumor-infiltrating lymphocytes and lymphocytes from tumor-draining lymph nodes does not yield a consistent picture, and does not allow conclusions to be made about the in vivo role of NK cells in humans.

The Chediak-Higachi syndrome in humans represents a condition similar to the beige mutation in mice. There is a drastic reduction in NK activity in these patients (61,99), and a concomitant increase in the incidence of neoplasia, again primarily of

lymphoid origin (37). Patients with severe combined immune deficiency present a similar picture (130,146). These cases provide a somewhat clearer correlation between NK activity and the incidence of tumors.

Another role that NK cells may play in the control of neoplasia is to serve as precursors of lymphokine-activated killer cells (LAK). Lymphokine activated killer cells appear to be distinct from resting NK cells in that they are an interleukin-2 expanded population of cytotoxic lymphocytes with a broader lytic capability and a more heterogeneous phenotype (116,138 and refs. within). There has been some controversy concerning the nature of the precursor cell for LAK activity, based mostly on interpretation of cell surface markers. However, it has become increasingly clear that NK cells can function as LAK precursors, although perhaps not exclusively. The cumulative data from mouse, rat and human studies indicate that LAK cells derived from the blood and bone marrow of these species display morphologic and phenotypic characteristics of NK cells (74,116,139,198,211). In the human, this includes LGL morphology, and a $CD3^-/NKH1^+/CD16^+$ phenotype (116,139,147). Rat lymphocyte populations depleted of NK cells serve as very poor sources of LAK activity, whereas purified LGL yield high levels of LAK activity (198).

Interleukin-2 is potent in its ability to augment the cytotoxic ability of the precursor population of lymphocytes and to support the proliferation of these activated effector cells. These LAK cells are important because they are able to kill freshly isolated autologous and allogeneic tumor cells, as well as tumor cell lines (53,117,138). This characteristic suggests that these cells may be more important physiologically than 'resting' NK in the control of tumor growth. The availability of

recombinant IL-2 has made it possible to produce enough LAK cells to attempt adoptive transfer experiments. This procedure has advanced to the stage where IL-2 and LAK have been used in the therapeutic treatment of cancer in humans (169), with some success in patients with melanoma, renal cell carcinoma, and certain other tumor types (reviewed in 138). Concerns about limiting the side effects and increasing the efficacy of the treatment regimen have led to the proposal that only purified NK cells be used in the production of the LAK effector population (116).

In summary, there is increasing evidence that NK cells may play a major part in the natural resistance to neoplasia, both in their role as lytic effector cells and as precursors for LAK.

Natural killer cells appear to interact with a variety of normal cells in mouse (159) and human bone marrow (65) and in the thymus (66). These cells are immature undifferentiated cells, and their ability to serve as targets has led to the hypothesis that natural killer cells have a regulatory role in normal hematopoiesis. This hypothesis was supported by the work of Cudkowicz and others, who described a radiation resistant lymphocyte in mice capable of inhibiting the graft of parental bone marrow into an F_1 progeny recipient (33, and refs within). Subsequently, it was shown that NK cells were the effectors responsible for this <u>in vivo</u> phenomenon (95). Later experiments by Warner and Dennert confirmed this observation, showing that reconstitution of mice with NK clones could prevent the take of a parental marrow graft in F_1 recipients (201). Further evidence of the capacity of NK cells to actively kill bone marrow cells came from studies measuring the clearance of intravenously injected, ^{125}IUdR-labelled bone marrow cells (159). As in the experiments measuring tumor take and metastasis, the rejection of parental marrow

grafts and the clearance of radio-labelled bone marrow cells correlated with the NK activity in the murine strains that were used. A parallel line of evidence exists in humans undergoing bone marrow transplantation. The likelihood of developing acute graft-versus-host disease is associated with a high level of NK activity prior to the transplant (114), and to the subsequent early reappearance of NK activity (41). NK activity often is enhanced in patients exhibiting conditions of depressed marrow function, most notably aplastic anemia (10) and in patients with NK lymphocytosis there often is an associated red cell aplasia (109) or neutropenia (143). However, attempting to draw conclusions about human NK function from this kind of correlation is necessarily simplifying a complex, multifactorial clinical picture.

In vitro experiments have not yet clarified the role of NK cells in the regulation of hematopoiesis. In some studies, purified LGL have enhanced the formation of erythroid burst forming units (BFU-E) from human peripheral blood and bone marrow (94), while in other studies inhibition of erythroid and myeloid colony formation has been reported (12,38,79). For example, the monoclonal antibody (9.1C3) is capable of blocking NK mediated cytolysis (87). A marked enhancement of BFU-E is observed when this antibody is added to cultures of unfractionated bone marrow cells (87). This result suggests an inhibitory role for NK. However, purified LGL and expanded LGL clones have been shown to produce colony stimulating factors (34,177). The apparent differential effects of NK on hematopoiesis may be a reflexion of the ability of NK cells to influence their microenvironment both by secreting soluble factors and through direct cell-cell contact. Thus, both inhibitory and stimulatory signals may be generated by the same cell population. Also, recent work has suggested that modulation of myelopoiesis by human LGL may depend on

the maturation stage of the progenitor (121).

The dual role of NK cells in the modulation of hematopoiesis, and as effectors limiting the development of tumors may be especially important in an organ like the bone marrow where both differentiation and proliferation occur. Aberrant hematopoietic cells that escape the control of regulatory systems (including modulation by NK cell products) and threaten homeostasis subsequently may be recognized as aberrant and be killed by NK effectors. It follows that one of the ways leukemia may develop is for these early aberrant cells to escape modulation and/or detection by NK cells, possibly through changes in their cell surface. In addition to their role in the control of tumor growth, as precursors for LAK, and in the regulation of hematopoiesis, NK cells have acquired many other functions. A summary of the proposed functions for NK cells in vivo is shown in **Table 1**, based on data derived from experiments in both human and animal systems.

Table 1. Functions of Natural Killer Cells

(adapted from 156)

1. Control of tumor cell growth
   - inhibit the development of primary tumors
   - control the development of metastases

2. Precursors of lymphokine-activated killer cells (LAK)

3. Control of hematopoietic stem cell growth and differentiation in:
   - bone marrow
   - thymus
   - spleen

4. Immunoregulatory properties:
   - production of cytokines
   - regulation of the antibody response
   - regulation of cell-mediated immunity
   - natural suppressor cells

5. Involvement in allograft rejection:
   - bone marrow transplantation
   - hybrid resistance
   - organ transplantation

6. Involvement in disease states
   - development of graft-versus-host disease (GVH)
   - contribution to some forms of aplastic anemia
   - potentiate autoimmune and neurological disease
   - contribute to the development of some forms of diabetes
   - involvement in various gastrointestinal disorders

7. Control of microbial infections
   - viral infections
   - intracellular and extracellular parasites
   - fungi
   - bacteria

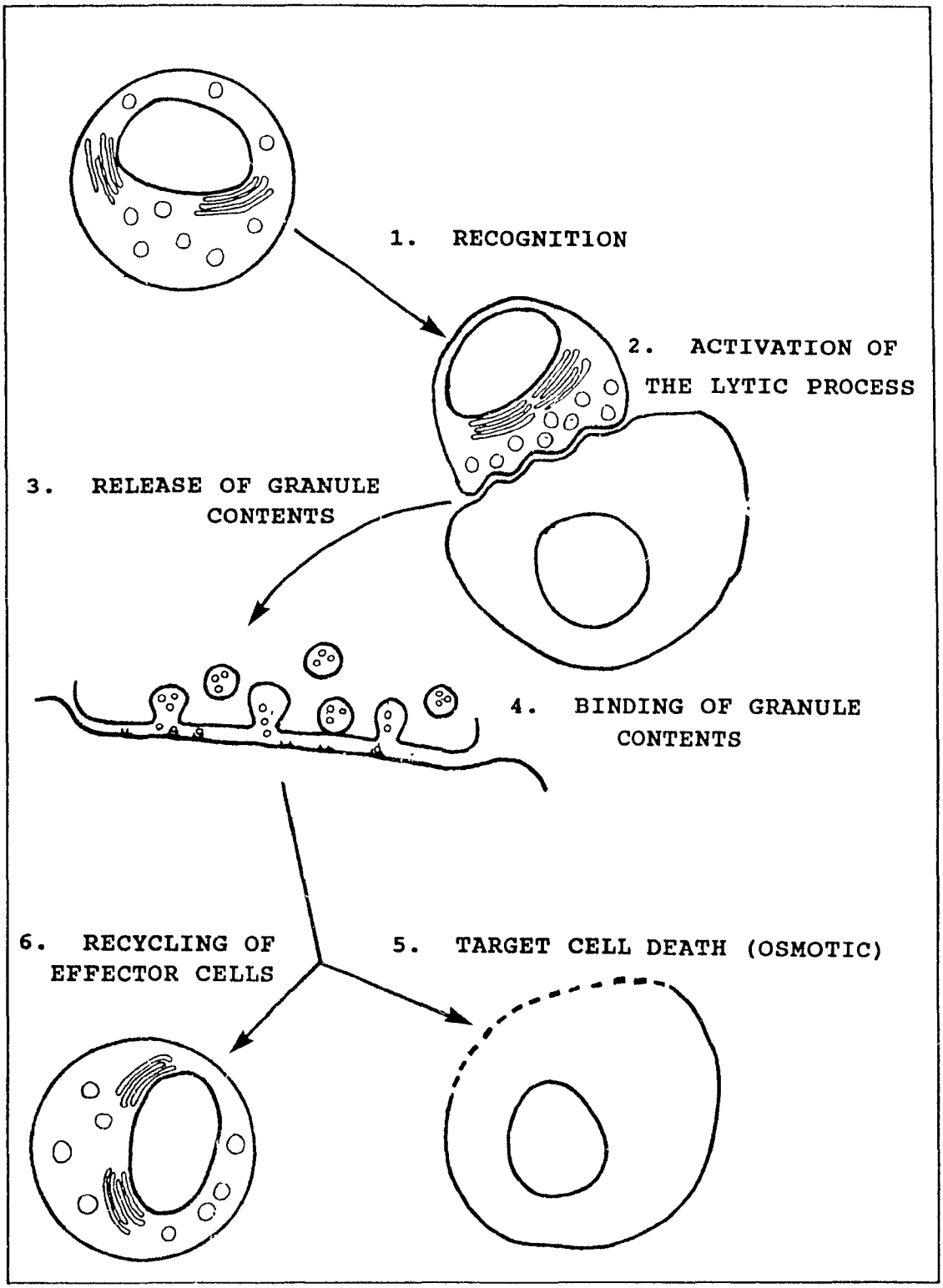
MECHANISMS OF CYTOTOXICITY

The nature of the interaction between natural killer cells and susceptible targets is a central issue in the definition of the NK cell, the characterization of its cytotoxic function, and the specificity of this function. Despite intense research effort, little is known about the nature of the molecules involved in the effector-target interaction cascade. However, a model that divides the effector-target interaction into several intrinsic phases has been proposed based on accumulated information. This model is represented in **Figure 2.**

The sequence of events depicted in **Figure 2** is essentially an elaboration of the stimulus-secretion model, whereby initial recognition of the target is followed by directed emiocytosis of cytoplasmic granule contents (26,163,165). The cytolytic nature of the granule contents further characterizes a primary function of the natural killer cell (215).

The initial step in the sequence of events is the recognition of, and subsequent binding to the target cell. This is followed by activation of the lytic process: an increase in intracellular free calcium concentration (43), possible involvement of the phosphatidylinositol response (205), reorientation of the microtubular organizing

Figure 2. Directed-exocytosis model of NK-mediated cytolysis.



complex (101), rearrangement of the granules (210), and polarization of the Golgi apparatus towards the target cell (29,101). The cytoplasmic granules fuse with the cell surface membrane of the natural killer cell at the point of communication with the target cell, releasing their contents into the intercellular space (210). There also may be active secretion of cytolytic molecules independent of those packaged in granules. The lytic molecules secreted and released from the granules then interact with the target cell membrane, perhaps through both nonspecific and receptor-mediated mechanisms. Some target cell-dependent activation of lytic molecules also may take place. Disruption of the target cell membrane leads to cell death (215). Once the natural killer cell has released its granule contents, it has the potential to recycle and begin the cytolytic process anew.

It is presumed that the initial recognition step is central to the expression of specificity by the natural killer cell; without effector cell recognition of a target cell there cannot be subsequent cytolysis. While this point is justified, and while recognition may serve as a prime regulation point, specificity may be expressed at a number of steps in the cytolytic sequence. The natural killer cell may recognize specific molecules on the cell surface of susceptible target cells. In turn, specific molecules on the target cell membrane may be required to activate the natural killer cell, which can then proceed with the cytolytic process. Once the lytic molecules have been released, they may have to bind to specific molecules on the target cell membrane, and they may have to be altered or activated before they can inflict damage to the target cell membrane. One consequence of this complicated picture is that natural killer cell specificity may be a reflection of the sum of these individual specificities and may involve a number of molecules on the cell surface of both

effector and target cells. Studies of the molecules involved in the process of target cell recognition must acknowledge this apparent complexity and should not focus simply on one species of molecule in isolation of the others.

Recognition of target cells

The effector-target binding step is the first in the sequence of events that ultimately may lead to the death of the target cell. It is the most obvious step where expression of specificity can be displayed, and where modulation may lead to tumor target escape from recognition. A central issue in the study of natural killer cell function is the nature of the recognition structures present on susceptible target cells. This issue remains unresolved and has hindered progress in the understanding of all aspects of NK physiology.

A number of different approaches have been used in attempts to define NK-target interactions, and some general characteristics of target cell recognition have been described.

A wide variety of normal (65,135,159), virally infected (18,124), and syngeneic, allogeneic, and xenogeneic tumor cells (reviewed in 137) are lysed by natural killer cells. However, there is some specificity in target cell susceptibility to NK cells; many cell lines and cell types are clearly resistant to NK-mediated lysis. In general, undifferentiated cells are more susceptible to NK-mediated lysis than are mature cells, although there are many exceptions (72,73,137 for review). Furthermore, there appears to be some heterogeneity in the specificity of target cell recognition by NK

cells. This observation is supported by three types of evidence. First, cold-target inhibition assays indicate which target structures are shared among different target cells. In these assays, a variation of the ^{51}Cr-release cytotoxicity assay, varying numbers of unlabeled NK-sensitive target cells are added to a mixture of effector cells and labeled target cells. Information about the identity and relative density of recognition structures between two target cells can be inferred from their competition patterns. Results from this type of experiment indicate that there may be a number of different target stuctures recognized by subsets of NK cells (181). Second, cellular immunoadsorption studies use monolayers of NK-sensitive target cell lines to deplete target-specific NK cells from the lymphocyte population (84,148). Analysis with a number of different target cell lines have confirmed the results with the cold-target competition assay, and have indicated that at least seven different NK specificities might exist (148). Third, interleukin-2 dependent NK clones have been used in an attempt to definitively describe the heterogeneity of NK target recognition. Natural killer cell clones typically are NK cells of a predetermined phenotype that have been clonally expanded and maintained in long-term continuous culture in the presence of a supporting concentration of interleukin-2 (6,137,161). NK clones differ from classic activated NK cells in that activated NK are merely resting NK cells whose activity has been activated or boosted by short-term exposure to an effective activating substance (IFN or IL-2, for example). Some of the NK clones do display selective reactivity against some NK-sensitive targets, and some clones are able to lyse target lines that are resistant to fresh NK cells, suggesting that sensitivity to NK cells is a only a matter of degree (6,78,137,161). These data appear to support the heterogeneity of both NK cell specificity and NK target structures,

and most studies assume that NK-clone specificity accurately represents NK specificity. However, NK clones are IL-2 dependent, and therefore may be defined as a form of activated NK. There is little evidence to support the belief that resting and activated NK share the same target cell specificities. Many NK clones exhibit a broad reactivity, not unlike activated NK. Also, many NK clones display unstable reactivity, many are not cytotoxic against any of the target cells tested, and many exhibit a cell surface phenotype uncharacteristic of resting LGL. Despite the technical advantages of using NK clones, these observations call into question their suitability as being representative of resting NK cells, and perhaps also of IFN and IL-2 augmented NK.

Several approaches have been used in attempts to further define the molecular nature of NK target structures. These include modification of the target cell surface with various enzymes, chemicals and biological agents, chemical induction of target cell differentiation, studies correlating the expression of certain target cell surface molecules with NK susceptibility, and attempts at blocking effector-target binding with simple sugars, glycosides, and target cell membrane components. Evidence linking the recognition of NK-susceptible target cells with any specific cell surface determinants has been indirect and necessarily inconclusive. Treatment of target cells with proteases decreases their susceptibility to NK cells, suggesting that the target structures are membrane proteins (165,166). Investigation of the glycosylation requirements of NK target structures has not proven a role for N-linked oligosaccharide components in NK target recognition. Simple sugars, phosphorylated sugars, and glycosides have been added to assays of NK function in attempts to define a role for specific carbohydrates (2,4,118). The concentrations of

carbohydrates required to detect inhibition are quite high, and most inhibitory effects are seen at post-binding stages in the lytic process (77,197). Treatment of NK-sensitive K562 target cells with a[illegible]ism is followed by an increase in their sialic acid content and a parallel decrease in NK sensitivity (14). An inverse relationship between neuraminidase releaseable sialic acid and NK sensitivity also has been reported in the mouse (212). This type of study suggests that sialic acid residues are involved in determining sensitivity to NK cells. However, as with other carbohdrates, sialic acid and sialo-oligosaccharides were shown to inhibit most effectively at a post-binding stage (195).

The use of cell line mutants with induced defects in glycosylation, and studies using inhibitors of oligosaccharide processing have led to conclusions that NK cells recognize target cells depending on the type of oligosaccharides presented at the cell surface. A wheat germ agglutinin (WGA) resistant variant of the metastatic/NK-resistant murine lymphoma cell line MDAY-D2 becomes NK sensitive (129) and also less metastatic (35). This shift in phenotype correlated with the appearance of premature truncation products of more complex oligosaccharides (36). These incomplete oligosaccharide complexes were found to be a mixture of bi- and triantennary, Concanvalin A (Con A) binding structures, terminating in N-acetylglucosamine residues. One conclusion from these studies was that N-linked oligosaccharides, including high mannose and some incomplete complex structures, may be recognized by NK cells as part of glycoprotein target structure. Similar results have been reported by Ahrens and Ankel (3,4) using a series of lectin resistant mutants of Chinese hamster ovary cell lines. Sensitivity to untreated or lymphokine-boosted NK cells and the ability of specific oligosaccharides to block NK

lysis were both linked with high-mannose type structures. Similarly, Pospisil et al (153) have defined triantennary oligosacharrides in some conformations which block pig NK activity against K562 target cells. However, it may not appropriate to equate changes in NK-sensitivity with altered NK-recognition, based solely on data from the chromium-release assay.

A number of NK-susceptible target cell lines can be induced into limited differentiation by various chemical agents; hemin (40,203), bleomycin (59), sodium butyrate (40,203), cytosine arabinoside (59), and phorbol esters (203). Although the extent of differentiation often was correlated with a decrease in NK-sensitivity, supporting the view that NK activity is predominantly directed against immature cells, differences in growth rate upon differentiation may have influenced the changes in susceptibility (59).

Several molecules have been proposed to be involved in NK-target recognition, based either on experiments correlating cell surface expression of a specific molecule with NK susceptibility, or on inhibition of target cell recognition with specific antibodies and specific molecules. Examples include stage-specific embryonic antigen one (SSEA-1) (68,223), class I histocompatability antigens (88,149), ganglioside G_{M2}(7), and transferrin receptor (TfR) (11,196). The work investigating TfR involvement in NK-target recognition is in many ways representative of this approach. Initial studies showed a correlation between cell surface expression of TfR and both NK susceptibility and the ability to compete against K562 target cells (K562 cells express high numbers of TfR during exponential growth) in cold cell competition assays (196). It was also apparent that TfR fragments and transferrin (Tf) saturated with iron could inhibit NK-target interactions (11). Alarcon et al (5)

have reasoned that if TfR was an NK target structure it could interact not only with transferrin as the natural ligand, but with an NK cell receptor sharing similar tertiary structure to transferrin. Their experiments indicate that 1) LGL treated with an anti-human-Tf antibody lose much of their lytic activity against K562, 2) 80% of LGL immunoselected on anti-human-Tf coated plates reacted positively with Leu-11 antibodies, and 3) the ability of LGL membrane extracts to block NK activity against K562 could be removed by pretreatment of the extracts with anti-human-Tf antibody. Although together these data imply a possible target structure function for TfR, this issue is not without controversy. Were TfR an effective target structure, modulation of TfR expression on NK-susceptible cells should affect their sensitivity. Dokhelar et al (39) measured NK sensitivity of K562 cells during exponential growth phase where TfR are expressed in high numbers, plateau growth phase or hemin-induced differentiation where TfR are significantly reduced, and after treatment with cycloheximide or actinomycin D which abolishes TfR expression. Significant changes in NK-sensitivity were not observed. Blocking the TfR active site with the monoclonal antibody 42.6 or with transferrin did not affect NK activity against K562 (39). Similar results were obtained by Bridges et al (24). They induced a twofold increase in K562 TfR by treating the cell culture for 24 hours with the iron chelator desferrioxamine, and a twofold reduction of TfR by 24-hour treatment with hemin. Again, changes in NK sensitivity were not observed. Furthermore, several types of target cells selected for similarity of TfR expression exhibited a wide range of sensitivities to NK cells and varied in their ability to compete in cold-target inhibition assays (24). Zanyk et al (222) used phorbol esters to induce changes in the TfR expression of target cells, and also concluded that TfR

expression alone does not determine NK sensitivity. Three monoclonal antibodies specific for the human TfR have been used by Lopez-Guerrero et al (115) to define functional epitopes on the TfR molecule. Antibody FG 2/12 blocked transferrin binding to the cellular receptor, whereas FG 1/5 and FG 1/6 did not. FG 2/12 inhibited NK activity (cytolysis and conjugate formation) of $CD3^-$ LGL against HeLa and Molt 4 target cells, but not against either K562 or U937, whereas FG 1/5 was effective only against HeLa targets, and FG 1/6 was ineffective against all targets tested. The authors concluded that NK cells recognize an epitope different but close to the transferrin binding site, and that although TfR is a target structure for NK cells, it may function only for some target cells. This last point would help explain the contradictory reports on the correlation between cell surface TfR expression and sensitivity to NK-mediated cytolysis.

The notion that inappropriate expression of a receptor linked to proliferation could lead to the recognition and killing of a cell is appealing, although recognition may not necessarily lead to target cell lysis. Natural killer cells may only partially recognize cells with increased expression of certain types of TfR, but may require an additional structure in order to complete recognition of a inappropriate cell and proceed with the cytolytic cascade. This proposition is supported in part by the demonstrated heterogeneity of TfR (115,191) and by experiments using mouse L-cells transfected with human TfR cDNA (131). These human-TfR-positive transformants are not susceptible to lysis by NK cells, but are effective competitors

The relationship between the cell surface expression of major histocompatibility complex (MHC) molecules and susceptibility to NK is unusual in that it is the lack of expression of these molecules on target cells that has been correlated with NK

sensitivity (88,149). Consequently, it has been postulated that NK cells may function as a defense system against tumor cell variants lacking cell-surface MHC molecules (88). This defense system would thereby complement CTL-mediated immunity, which must recognize MHC molecules to kill a target cell. Evidence supporting this hypothesis comes from experiments in both mouse and human systems. Piontek et al (149) selected H-2 deficient variants of the murine lymphoma YAC-1 and showed that these sublines became highly NK-sensitive. These, and other H2 loss variants exhibit a less malignant phenotype when injected into syngeneic host animals (90). Further characterization of these variants has indicated that despite three distinct defects in H-2 expression all result in the NK-sensitive/less malignant phenotype (112). Similar variants have been derived from human T and B-lymphoblastoid cell lines, the loss of Class I but not Class II HLA expression correlating with increased NK-sensitivity and conjugate formation (67,180). The human cell line Daudi is HLA^- and has been transfected with cloned human beta$_2$-microglobulin DNA that allows these cells to express Class I molecules on the cell surface (155). The Daudi HLA Class I^+ transfectants showed a modest but increased resistance to NK and LAK-mediated lysis. However, transfection of HLA K562 cells with human HLA Class I DNA has produced contradictory results. K562 transfectants with varying cell surface Class I expression did not show any changes in NK-sensitivity or competitiveness in cold target inhibition studies (106). Although the data supporting loss of histocompatibility antigens as a determinant of target susceptibility in mouse is compelling, a different mechanism may be predominant in humans. Regardless, it has been suggested that H-2/HLA expression may only be influential at post-binding stages in the lytic cascade (111,113).

A more direct approach to the identification of possible NK target structures has been isolation of membrane components from NK-susceptible target cells and the study of their effects on the binding of NK cells to target cells. Roder, et al (162,167) reported in 1979 that high molecular weight (130-240 kDa), Concanavalin A-binding glycoproteins eluted from SDS-PAGE gels could inhibit NK-target conjugate formation. Further characterization of these inhibitory molecules has not been reported. Obexer, et al (133) and Henkart, et al (69) have reported similar findings using purified LGL in the inhibition assays and improved techniques of membrane protein isolation and fractionation. Once again, the inhibitory molecules were glycoproteins capable of binding to Concanavalin A and covered a wide molecular weight range; 10 kDa and 80-200 kDa from Obexer, 30-165 kDa from Henkart. It is interesting that Henkart could achieve significant inhibition of LGL-target formation only if the isolated membrane glycoproteins were reconstituted into micelles of endogenous membrane lipids. This suggests the involvement of a number of membrane components in NK-target recognition. Again, there has not been any further characterization of these molecules since they were initially reported.

Despite considerable research effort, the molecules involved in target cell recognition remain unknown. The study of target structures and the ability to gain useful information has been hampered by the lack of a definitive and objective assay to measure recognition, by a reluctance to accept that the recognition event might involve a complex of molecules on the cell surface and not just a single target molecule, and by the inveterate belief that any specific receptor or target structure of consequence must be glycoprotein.

Activation or triggering of cytolysis

Once a natural killer cell has recognized and bound to its target, it must in turn be triggered to lyse that target. The reorientation of the microtubules, Golgi apparatus and cytoplasmic granules is a part of this process, and only occurs in NK cells that interact with susceptible targets (29,101). Modulation of the NK target K562 with IFN renders it less sensitive to NK-mediated lysis, and also defective in its ability to induce both reorientation of the Golgi and increases in intracellular calcium in NK cells (54). These ultrastructural changes seem to be required before lysis can occur. Increases in intracellular free calcium (43), and an increase in the metabolism of several cyclic nucleotides and the phosphotidylinositols (PI) (178,205) have been observed following effector-target interaction and may play a role in linking recognition to cytolysis. This idea is supported by the observations that natural killer cytotoxic factor (NKCF) (51), and perhaps perforin (216) can be released from NK cells by stimulation with the phorbol ester TPA and calcium ionophore, a combination that mimics the physiologic consequences of increased PI turnover.

The requirement of a triggering step in the cytotoxic mechanism also has been suggested by studies using various inhibitors. For example, inhibitors of serine-dependent proteases and/or sulphydryl-dependent enzymes inhibit the release of NKCF, but not effector-target binding, or target cell susceptibility to NKCF (20). This suggests that triggering of NKCF may be mediated by a proteolytic event.

Antibodies against the human equivalent of murine 200/Ly-5 (94,132), and inhibitors of arachidonic acid lipoxygenation (22) block NK-mediated K562 lysis at the post-binding effector cell level, indicating a role for these molecules in the intermediate steps of the lytic sequence. In a series of experiments using a calcium pulse technique in combination with a dextran suspension assay and 51Cr-release, Hiserodt, Targan and colleagues used a series of inhibitors to further dissect the stages in the mechanism of cytotoxicity, and their cation requirements (80,81). However, the concentrations of some of the inhibitors (verapamil, DMSO, EGTA) far exceed those required to ensure specificity and minimize effects on membrane integrity, intracellular pH, and intracellular calcium metabolism. These limitations call into question the conclusions made from these types of experiments.

Lytic molecules and their interaction with target membranes

The stimulus-secretion model of NK-target interaction implies that information about the nature of the cytolytic effector molecules may be found by examining the granule contents of the natural killer cell. Support for this hypothesis comes from studies that point to degranulation as a necessary prerequisite for target cell lysis. Natural killer cell activity in humans and mice can be inhibited by treatment of NK cells with monensin (28,29), which blocks the intracellular transport of newly synthesized molecules from the Golgi, with degranulation inducing agents (127,128), or with lysosomotropic agents (175). Ultrastructure studies also have indicated that degranulation is a consistent feature in the cytotoxic mechanism of NK cells

(reviewed in 29). The isolation of cytoplasmic granules from LGL and the demonstration of their potent ability to lyse cells have established granule-release as the likely mechanism for delivering cytotoxic molecules (215).

The cytoplasmic granules of natural killer cells have been shown to contain three serine esterases (human granzymes 1,2 and 3) (64), a T- and natural killer cell-specific trypsin-like serine protease (45,48), perhaps another unique serine esterase (BLT-E) (126), protease-resistant proteoglycans of the chondroitin A type (119), and cytolytic pore-forming protein or perforin (215), all of which may take part in the killing of a target cell. As well, a range of other molecules has been described whose functions in the killing process are not clear. These include beta-glucuronidase (55), arylsulfatases (276), lipo-oxygenases (82), and a urokinase type plasminogen activator (27). There is no evidence that all of these molecules reside in the same type of granule, and are not functionally segregated within the cell.

Perforin. Perforin has been isolated and purified from the granules of rat, mouse and human Il-2-dependent NK and CTL derived cell-lines (70,110,220,215), its cDNA sequence and deduced amino-acid sequence have been described (108), and its function has been well characterized. Perforin is a single protein species with a molecular mass of 70 kDa under reducing conditions (215). The perforin monomers, once released from the effector cell, bind to and insert into the lipid bilayer of the target cell where they polymerize into macromolecular tubular complexes or pores (215,217). Only perforin monomers can insert into a membrane, and polymerization must take place entirely within the membrane for them to be lytically active. As well, the polymerization of perforin monomers is strictly

calcium-dependent (215). These characteristics insure that perforin monomers escaping into the surrounding extracellular fluid polymerize quickly and therefore are rendered incapable of inserting into, and damaging the membranes of non-target cells.

The pores formed by the progressive aggregation of perforin monomers are functional, displaying a large unitary conductance and a high voltage-resistance (215,219). Target cells appear to depolarize rapidly, undergo an influx of water, and leakage of electrolytes and some macromolecules (215,218). The cells swell and ultimately burst.

Perforin does not appear to display any intrinsic specificity for type of target membrane. Although an intact effector cell containing perforin-rich granules displays target cell specificity, the isolated granules or the purified perforin are able to kill a wide range of cell types, and exhibit activity against liposomes and reconstituted planar lipid bilayers that would not be sensitive to the intact NK cells (110,215,218,219).

Considerable evidence has accumulated relating perforin to complement proteins, particularly those of the membrane attack complex (MAC). The MAC is the name given to the C5b-C9 group of complement proteins that is responsible for membrane disruption and transmembrane tubule formation. Perforin and the complement protein C9 share many characteristics including molecular mass, unit conductance and electrical characteristics of the pores formed, functional size, and immunologic cross-reactivity (214). Anti-C9 antisera have been used in the affinity purification of perforin from human lymphocytes, and have been shown to block killing of K562 target cells by human LGL (220). Ultimately, description of the

cDNA sequence of human perforin has revealed 17-20% homology with the human complement proteins (C7,C8,C9) that form the transmembrane channel of the MAC (108). This relationship between perforin and complement proteins has raised the issue of homologous restriction. Natural killer cells and CTL, as well as other nucleated cells, are resistant to lysis by homologous but not heterologous complement (85). It appears that the activity of perforin also may be restricted in this manner, thereby protecting functional effector cells from self-inflicted damage. A soluble form of homologous restriction factor has been isolated from the cytoplasmic granules of cultured human LGL (221). However, it also has been shown that NK and CTL are resistant to lysis by both homologous and heterologous perforin (85). This suggests that the protective mechanism associated with complement and with perforin are different. Regardless of this controversy over the nature of the protective effect, it is clear that the cytotoxic cells that use perforin as an effector molecule are protected from lysis, and are able to recycle.

Most of the biochemical and functional studies have used IL-2 dependent NK-derived cell lines or clones as a source of isolated cytotoxic granules and purified perforin. These cytotoxic cells are by definition activated, and may not represent primary or resting NK cells. This view is supported by studies that failed to detect the complement-like perforin pore structures on lysed targets or the presence of cytolytic granules and perforin in freshly harvested, unactivated cytotoxic cells (15,218). Moreover, the process of IL-2 activation and cell line production has been shown to lead to the acquisition of cytoplasmic granules, and the development of perforin staining and activity (64, for example). It appears that the expression of perforin can be induced by IL-2, and therefore may be predominantly a characteristic

of activated cytotoxic cells.

Further evidence disputing a predominant role for granule perforin in lymphocyte-mediated killing comes from studies using cytotoxic T-lymphocytes (CTL) as effectors. Although initially calcium was said to be necessary for the CTL lethal hit (120), and although perforin-mediated target membrane damage is entirely calcium dependent (215), a reexamination of this relationship has suggested that calcium dependence may be dictated by target cell type (141,188) and also by the different CTL populations used (83). For example, Ostergaard and Clark have shown that the antigen-specific CTL clone KB1.24 can lyse EL-4 targets in the absence of extracellular calcium, whereas B-6 blasts require the presence of calcium in order to be lysed (141). In general, antigen-stimulated, Il-2-dependent CTL and primary CTL are able to kill some targets in the absence of calcium; cloned CTL lines that behave like LAK cells can kill all targets in the absence of calcium, murine LAK appear to require calcium for all target killing (30). Other studies have shown that CTL clones are able to lyse target cells in the complete absence of extracellular calcium, and under conditions where intracellular calcium stores have been depleted (141). The secretion of cytoplasmic granules is entirely calcium-dependent, and therefore should not be possible under these conditions.

Similar studies have not been performed with natural killer cells. However, antibodies raised against human perforin apparently are able to block both ADCC (200) and spontaneous cytolytic activity of human LGL (220). Antibodies raised against the cytoplasmic granules of rat LGL tumor cells also block NK and ADCC activity, but are not effective against CTL activity (157).

Despite convincing evidence supporting perforin as the lytic mediator in cytotoxic

cell lines (CTL and NK-derived), the role of perforin in vivo remains unclear. It is becoming more apparent that cytotoxic lymphocytes may possess more than one type of lytic mechanism.

NKCF. NK cells release natural killer cytotoxic factor (NKCF) after they have been incubated with susceptible target cells (206). The active component is a protein of apparent molecular weight of 15-40 kDa (17,20,171), and appears to require both disulphide bonds and its oligosaccharide chain for lytic activity (20). NKCF may consist of more than one species of cytotoxic molecule (20,171). It has not yet been determined whether NKCF is present in the granules of unactivated NK cells or if emiocytosis is the mechanism of its release from NK cells. Treatment with monensin or Sr^{2+} has been shown to interfere with the secretory process of cells, Sr^{2+} by causing a degranulation of the cell (128), and monensin by interrupting the transport of newly synthesized membrane proteins and secretory molecules from the Golgi apparatus to the cell membrane (52). Both of these agents also inhibit NK activity (29,127), and both have been shown to inhibit NKCF production (19). However, granules from NK-derived cell lines have not been shown to contain NKCF, although the presence of significant amounts of perforin in these granules may mask the activity of NKCF. The small percentage of LGL in peripheral blood has not permitted isolation of enough granules for adequate analysis.

NKCF-mediated and perforin-mediated cytoxicity differ from one another in a number of characteristics. The stimulation of NKCF release seems to be rather nonspecific. Some target cells that bind NK effectors but are resistant to lysis have been shown to stimulate the release of NKCF (207,208,209). Although the release step may be nonspecific, NKCF itself demonstrates inherent specificity. Cell-free

supernatants containing NKCF are selectively cytolytic for NK-sensitive target cells (206,207). Therefore, it has been proposed that the majority of NK target specificity (with target cell death as the end result) may be determined by target sensitivity to NKCF, most likely through NKCF-specific membrane binding sites. In contrast, perforin is presumably released upon direct interaction of NK cell receptors with specific structures on the target cell membrane. Once released, perforin displays little specificity in the membranes with which it interacts.

Subsequent to its interaction with a specific binding site, NKCF may require an activation step before it can inflict damage to the target cell (20). Perforin activity can be enhanced by the simultaneous secretion of a 60-kDa serine protease (126), but this does not represent an essential activation process and is not dependent on the serine protease activity of the molecule.

It is not yet known how NKCF mediates lysis of target cells. Further, insight into the biochemical pathways involved will require the more rigorous purification and characterization of the NKCF molecules. Overall, biochemical information has remained elusive beyond the initial description of NKCF.

The possibility of natural killer cells possessing two distinct cytolytic mechanisms raises the issue of their relative roles in mediating NK function. There is no clear evidence revealing either NKCF or perforin as the predominant lytic mechanism in unactivated NK cells. It has been observed using a double conjugate modification of the single cell assay that a single LGL can lyse both NK and ADCC targets simultaneously (21). If NKCF is the predominant lytic molecule, then a mechanism for sidestepping the target specificity of NKCF must exist in order for the ADCC target to be killed. Thus far, this has not been addressed. Perforin, once released,

would not have to overcome any specificity barriers. However, perforin cannot be immunolocalized in the granules of unactivated NK (218). This may represent an inability to physically detect minute yet functional quantities of perforin. Alternatively, the NK cell may be capable of a sufficient level of intracellular organelle sorting to allow the cell to specifically direct two distinct lytic mechanisms. Evidence for the independent degranulation of two different granule fractions exists for CTL effector cells. Clark et al have shown that the lytically active CTL cell line L3 (with demonstrable perforin-containing granules) is not able to lyse CD3-derivatized red blood cells, although they have measured proteoglycan and serine esterase release from these CTL effectors (30). If perforin is found in the same granules as serine esterase and proteoglycans, then the CD3-RBC-induced degranulation of the CTL effectors should have led to lysis of these targets. This dissociation of the lytic and degranulation events in CTL supports the hypothesis that different populations of granules may exist within the cell and may be under independent control. Precedent for this mechanism is found in cells of the anterior pituitary where growth hormone and prolactin are packaged in separate granules (46), and in primary versus secondary granule release in human neutrophils (107).

Antibodies raised against perforin have been shown to block both the NK and ADCC activity of LGL (200,220). This type of antibody will block also the activity of NKCF (19,77). The reasons for this apparent cross-reactivity have not been determined.

Serine proteases. A number of serine proteases have been isolated from the granules of NK-derived cells. Those with identified catalytic activity have neutral pH optima, suggesting that they may be most active after exocytosis (64). They are not

directly lytic to target cells, and may be involved in the activation, release or modulation of cytotoxic molecules (126). Protease inhibitors do inhibit the release of NKCF, implying a role for serine proteases.

Proteoglycans. Protease-resistant proteoglycans of the chondroitin A type have been shown to be present in NK cell-derived cytoplasmic granules, and are actively exocytosed during contact with NK-sensitive target cells only (119,173). A number of functions have been proposed whereby proteoglycans may indirectly participate in the cytolytic process (192). Proteoglycans may facilitate transfer of cytolytic molecules to target cells. However, pure perforin is as effective as intact granules, which suggests proteoglycans are not required. Proteoglycans may be involved in the packaging and condensation of cytoplasmic granules, and may protect the effector cell from autolysis by inactivating cytolytic molecules within granules. The latter possibility is supported by observations that proteoglycans bind and inactivate perforin, especially at the low pH present within cytoplasmic granules (193).

In summary, research over the past ten years has confirmed a role for natural killer cells as effectors and regulators in the immune system. Despite this progress, many areas of NK physiology remain speculative. It is essential that there be a better understanding of the molecular and biochemical nature of natural killer cell specificity before one can fully exploit them for therapeutic ends.

MATERIALS & METHODS

RESULTS

DISCUSSION

The three papers that follow represent the Materials and Methods, Results and Discussion sections of this dissertation. These papers are reprints of articles published in the established literature and subsequently will be referred to by the following labels:

Publication 1: Target Cell Specificity of Human Natural Killer (NK) cells I. Development of an NK-Resistant Subline of K562

Stephen L. MacDougall, Chaim Shustik and Arthur K. Sullivan

Cellular Immunology 76: 39-48 (1983).

Publication 2: Target Cell Specificity of Human Natural Killer (NK) Cells II. Apparent Change with Activation

Stephen L. MacDougall, Chaim Shustik and Arthur K. Sullivan

Cellular Immunology 103: 352-364 (1986).

Publication 3: Increased fucosylation of glycolipids in a human leukemia cell line (K562-Clone I) with decreased sensitivity to NK-mediated lysis

S. L. MacDougall, G. A. Schwarting, D. Parkinson and A. K. Sullivan

Immunology 62: 75-80 (1987).

The initial step in the interaction of an NK effector cell with a susceptible target cell is the formation of effector-target conjugates. This intimite intermembrane communication is thought to be specific, and to involve distinct membrane-associated molecules. Although a variety of experimental approaches have been used to try to gain insight into the nature of these molecules, they have not produced firm knowledge about the membrane characteristics that define sensitivity to NK-mediated cytolysis.

Our experimental approach to the study of NK-target interactions has been to raise NK-resistant variants of the standard NK-susceptible target cell line K562, and to compare these variants with the parent cell line in order to discern the biochemical nature of their resistance to NK-mediated lysis. The selection procedure for deriving NK-resistant variants was designed with the hope of limiting the basis of NK-resistance to the initial recognition events. This was attempted using a modification of an experimental method developed by Saksela et al (170), and described in the Materials and Methods section of **Publication 1**. The Saksela method used the formation of effector-target conjugates as a means of isolating the lymphocytes that bound to K562 target cells. Briefly, they mixed peripheral blood lymphocytes (containing NK effectors) with K562 target cells and allowed effector-target conjugates to form. This mixture was then layered onto a cushion of 17% Percoll and gently centrifuged, sedimenting the NK-K562 effector-target

conjugates and leaving the free lymphocytes and free K562 cells at the interface. The NK-K562 effector-target conjugates were collected, the conjugates were disrupted and the lymphocytes that had bound to K562 cells were isolated and subsequently characterized. Our modification of this approach sought to remove NK-K562 effector-target conjugates and to isolate K562 cells that did not form conjugates with lymphocytes. We reasoned that if some K562 cells did not form conjugates they may represent variant cells that were not recognized by the majority of NK lymphocytes, and therefore they may be resistant to NK-mediated lysis.

There are a number of advantages to our approach. First, and most importantly, NK-resistant cell variants selected in this manner allow direct biochemical comparison between cell lines (the K562 parent and an NK-resistant variant) that may differ only in their sensitivity to NK-mediated lysis. This circumvents many of the shortcomings of indirect comparison of unrelated cell lines. Second, the NK-sensitive/resistant cell line pair allows comparative analysis of a number of cell membrane components and their interactions. Third, the selection procedure does not involve treatment of the target cells with mutagens and can be considered an in vitro parallel to selection mechanisms that may operate in vivo.

Publication 1 describes the derivation of an NK-resistant subline of K562, and its initial characterization. The development of such an NK-resistant cell line and its differential sensitivity to resting and activated NK cells raises two important issues. First, what is the biochemical basis for the resistance to NK-mediated lysis, or conversely, what determines the NK-sensitivity of a given target cell? Second, is there a change in target cell specificity as NK cells are activated, and how may this

change in specificity manifest itself biochemically? **Publication 2** and **Publication 3** address these issues.

These three publications represent the deliberate development of a research approach to these two central and unresolved questions in NK cell physiology.

Publication 1: Target Cell Specificity of Human Natural Killer (NK) cells I. Development of an NK-Resistant Subline of K562

Stephen L. MacDougall, Chaim Shustik and Arthur K. Sullivan

Cellular Immunology 76: 39-48 (1983).

The following statement regarding experimental contribution and authorship is made in accordance with the "Guidelines Concerning Thesis Preparation, Section 7: Manuscripts and Authorship", Faculty of Graduate Studies and Research:

The ideas, experiments, and writing represented in **Publication 1** were produced by Stephen L. MacDougall, with the supervision and input of Arthur K. Sullivan as thesis supervisor. Chaim Shustik worked out the conditions for protein A activation of effector cells (Figure 5) and performed these initial experiments. All subsequent repeats and modifications were done by Stephen MacDougall.

CELLULAR IMMUNOLOGY 76, 39–48 (1983)

Target Cell Specificity of Human Natural Killer (NK) Cells

I. Development of an NK-Resistant Subline of K562

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By depletion of effector–target conjugates and cloning, a variant of the human leukemic line K562 that is partially resistant to lysis by natural killer (NK) cells when tested under conditions of culture and assay identical to that of the parent has been derived. Its karyotype shows markers similar to the original K562. The resistant phenotype remains stable after over 1 year of continuous passage and persists in multiple replicate subclones. However, it can be killed to a degree equal to the parent by antibody-activated complement, antibody-dependent cellular mechanisms, and by effector cells activated by staphylococcal protein A. These observations and experiments on cold target competition suggest that on its surface there is a significantly decreased, absent, or blocked effective target structure for a major population of unstimulated peripheral blood NK cells.

INTRODUCTION

In rodents and in man there appears to be a natural mechanism that may contribute to the control of hematologic and solid tumors (reviewed in (1, 2)). This phenomenon is mediated by a subpopulation of lymphoid cells that have the capacity to lyse *in vitro* a wide range of tumor-derived target cells (2). These same effectors may lyse also a small number of normal cells from the thymus and bone marrow (3, 4). Thus, it has been proposed that these "natural killers" (NK) may function to regulate hematopoiesis and to defend the organism against neoplasia in the early stages of growth.

The molecular mechanism by which NK cells recognize susceptible targets is unknown. Through studies using techniques of competitive inhibition among susceptible cells (5), depletion of effectors on immobilized targets (6) and direct observation of single target–effector conjugates (7), a multicomponent model has evolved that includes binding as an essential first step in the activation of the lytic mechanism (8). Within the murine system, others have sought to decipher the heterogeneity by developing resistant variants of susceptible target cell lines. Attempts to select resistants by serial passage *in vivo* has been inhibited by reversion once the cell line has been returned to culture *in vitro* (9). Others have reported stable resistant variants of murine lymphoma lines which appear to lack a surface recognition structure (10, 11). Collins *et al.* (12) have described a line, derived from transformed fibroblasts, which putatively has acquired resistance on the basis of an effector-inactivating mechanism. However, direct comparison of these studies must be made with caution since different systems

may be operating in the recognition of hematologic and solid tumors (13) At present, no similar model has been developed with human cells

We report here the characteristics of the first stable human leukemic cell line that has been made resistant to spontaneous lysis by peripheral blood lymphoid cells This derivative of the myeloid–erythroid leukemic line K562 (14) has remained phenotypically stable for over 1 year of continuous passage and through two further cycles of recloning

MATERIALS AND METHODS

Selection of variant line Target K562 cells were mixed with a twentyfold excess of fresh peripheral blood lymphocytes and incubated at 37°C in 5% CO_2–air atmosphere for 2 hr in a total volume of 6 ml Three milliliters of the suspension were then layered onto 3 ml of 17% Percoll and spun for 10 min at 50*g* as modified from Saksala *et al* (15) This procedure effectively sedimented all of the lymphocyte–K562 conjugates and left very few free K562 cells at various levels above the interface Layers of 0 5 ml were taken from above the interface and cultured in 24-well plates Those wells in which vigorous growth was observed were tested for sensitivity to natural lysis The cells from the least sensitive wells were then used to repeat the entire procedure After two cycles of conjugate depletion with the same effector cell donor, the most resistant of the sublines was incubated with fresh peripheral blood lymphocytes at a 20 1 lymphocyte target ratio and cultured at 37°C without further separation to allow maximum cytolysis Within 14 days after regrowth of unkilled targets, cells were passaged until debris was no longer observed At this stage, the subline was cloned by placing 0 2-ml aliquots of cell suspension at 1 cell/ml into each well of flat-bottomed 96-well culture plates All wells that grew up were tested for resistance and the most resistant was recloned Cell line 3C3 originated from the first cloning procedure and was the most resistant of the lines tested Clone I described below was obtained as a subclone of 3C3 All cells were grown in RPMI 1640 media with 10% fetal calf serum (FCS) and were passaged twice a week

Of note is the fact that we were not able to obtain stable resistant lines, without the binding step, by selection through regrowth after two cycles of exposure to effectors

Cell line and culture conditions The human tumor cells used as targets were the erythroid–myeloid line K562, the B-lymphoblastoid line Raji, and the K562 variant 3C3 or Clone I. Culture conditions for all of the cell lines were identical Cells were passaged two or three times weekly in RPMI 1640 supplemented with 10% FCS and kept at 37°C in a humidified 5% CO_2 incubator Peripheral blood lymphocytes for effectors were obtained from healthy donors by venipuncture The separation of lymphocytes from whole blood was by centrifugation on Ficoll–Hypaque barriers (Lymphoprep, Pharmacia) Lymphocytes were washed three times in RPMI–FCS and resuspended at the desired concentrations

For some studies, cultures of the Raji, Clone I, and K562 lines were passaged in RPMI–FCS supplemented with 24 μg/ml tylocine (Gibco) After 1–2 weeks of exposure to the agent, the cells were harvested and used as targets in the cytotoxicity assay described above.

Karyotypes were analyzed by standard methods of Giemsa binding by Dr. M Jean-Jacques Vekemans of the Prenatal Diagnosis Unit of The Montréal Children's Hospital

Antisera W6/32 (anti-HLA p44), EC-3 (anti-β_2-microglobulin), and anti-HLA-DR (DA-2) were a generous gift of Dr W Bodmer, Imperial Cancer Research Fund, London, England Fluorescent conjugates were obtained through commercial sources (Cappel Laboratories) Rabbit anti-K562 was obtained by immunizing New Zealand white rabbits on two occasions 2 weeks apart with 10^7 parent K562 cells suspended in complete Freund's adjuvant

Cytotoxicity assay. The cytotoxicity assay was modified from Pross *et al* (*16*) Target cells were harvested from suspension cultures in log phase of growth, incubated at 37°C for 1 hr with 100 μCi ^{51}Cr (New England Nuclear), washed three times in RPMI–FCS, and resuspended at the desired concentration Appropriate numbers of peripheral blood lymphocytes were mixed with 5×10^3 labeled target cells in 150 μl of RPMI–FCS in 96-well polyvinyl culture plates and incubated for 4 hr at 37°C in a humidified 5% CO_2 incubator After mild centrifugation, 75 μl of supernatants was recovered and counted in a gamma counter (Nuclear Chicago, Inc) Percentage specific lysis was calculated from counts per minute according to the following equation

$$\% \text{ specific lysis} = \frac{\text{sample} - \text{spontaneous release of } ^{51}\text{Cr}}{\text{maximum control} - \text{spontaneous release}} \times 100\%$$

Spontaneous release consisted of supernatant from wells lacking effectors Maximum control consisted of resuspended samples from replicate wells

For analysis of antibody-dependent cellular cytotoxicity, the target cells were radiochromium labeled as above, incubated with a 1 4 dilution of decomplemented rabbit anti-K562 antiserum, washed thrice in media, and assayed in a manner identical to untreated cells

Complement-mediated lysis was measured by incubating the cell mixtures for 90 min at 1×10^4 ^{51}Cr-labeled cells with 10 μl of antisera at the noted dilution and 10 μl of rabbit complement (Low-Tox-H Rabbit Complement, Cedarlane Laboratories Ltd , Hornby, Ontario, Canada) at a dilution determined for each individual lot

Cold cell inhibition Cold cell competitions were performed by a modification of that described by Masucci *et al* (17), using K562, Raji, or Clone I cells as cold competitors against labeled K562 or Clone I Appropriate numbers of unlabeled competing cells were mixed with 2.5×10^5 PBL as effectors in 100 μl RPMI–FCS (10%) in 96-well polyvinyl culture plates and incubated for 1 hr at 37°C in a CO_2 humidified incubator Labeled target cells, 50 μl at 1×10^5/ml, were then added to all wells Plates were incubated for a further 4 hr at 37°C and analyzed as above

Activation of NK cells. Lymphoid cells were activated as described by Catalona *et al.* (18) by incubation for 12 hr with 50 μg/ml of Staphylococcal protein A (Pharmacia) in RPMI-FCS at 37°C, washed three times in media, and used as effectors in the standard cytotoxicity assay

RESULTS

Analysis of Lineage Derivation

For the studies reported here, we used Clone I derived from Clone 3C3 These cells grow in suspension and by light microscopy appear identical to the parent K562 By fluorescence microscopy, they bear no surface immunoglobulin, β_2-microglobulin, HLA p44, or Ia-like antigens Karyotype analysis of K562, 3C3, and Clone I all show

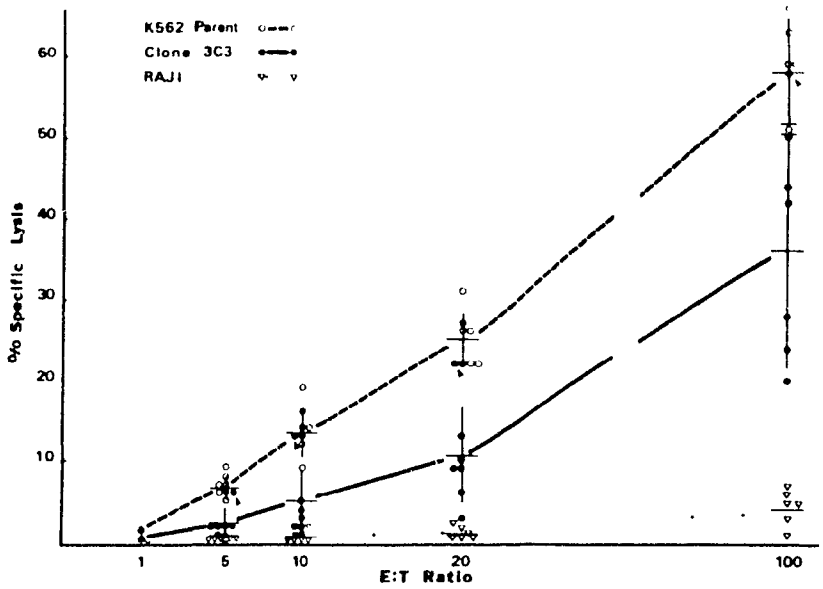

FIG 1 Natural cytotoxicity against cell lines by effector cells from seven subjects Each point represents the mean of triplicate values for each subject, the bars represent the overall mean ± 1 SD The arrows denote one subject whose activity against the resistant line was markedly different from all others, no similar finding was noted in over 20 other subjects At effector to target ratios of 5, 10, 20, and 100, the differences between K562 parent and Clone 3C3, and between 3C3 and Raji are all significant with $P < 0.001$ by Student's t test

a modal number of 66 chromosomes, and all possess the Philadelphia-like and E-group anomalies originally described (14) Thus, we conclude that these clones are derived from K562 and are not an unrelated contaminating line

Susceptibility to Lysis by NK Cells

Shown in the graph in Fig 1 is the specific lysis of parent K562, selected variant 3C3, and Raji cells plotted as a function of effector cell number Even at an effector/target ratio of 100 1, the K562 variant is significantly less sensitive to kill than is the parent, yet, at all points it remains significantly more sensitive than Raji. A similar pattern results from both the standard 4- and 12-hr assays This resistant phenotype has been stable when assayed several times over a period of 18 months using effectors from multiple donors A similar degree of resistance shown by subclones of 3C3 (Fig 2) is further evidence in support of the phenotypic stability of the variant.

Recent reports (19) have implicated mycoplasma in the activation of NK activity Although both parent and resistant lines were negative for mycoplasma as determined by staining with Hoechst 33258 (24), to minimize the possibility that the characteristics of the variant represents simply a mycoplasma-free status, we grew both lines in anti-PPLO media Upon retesting, the difference in susceptibility remained unchanged To assess this further by direct transfection and to eliminate the possibility that the variants may secrete a product that would mask the target structures, the parent and derivative lines were retested after two passages in supernatants in which the other line had been grown Again, the resistance of Clone I in the NK assay

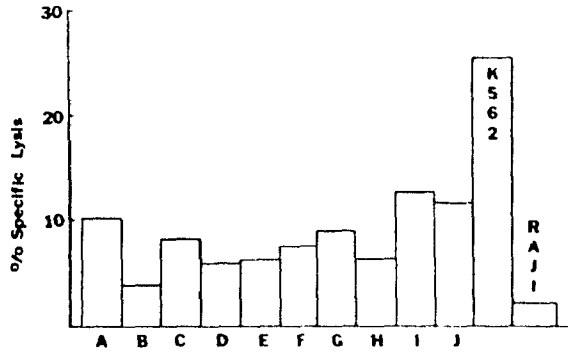

FIG 2 Lysis of subclones of 3C3 From 3C3 cells cultured at limiting dilution, ten subclones designated by letters A–J, were selected at random and assayed as in Fig 1 Each bar represents the specific lysis in comparison to the parent K562 and Raji lines Effector target ratio 20 1

remained unchanged (data not shown) On the basis of these experiments, we conclude that this line is a stable derivative resistant to NK-mediated lysis on the basis of an alteration intrinsic to the cell

Susceptibility of Clone I to Other Mechanisms of Lysis

Shown in Fig 3B is the titration curve of sensitivity of parent K562 and Clone I to complement-mediated kill by different dilutions of a rabbit anti-K562 antibody The superimposable curves indicate that the resistance of Clone I is due not merely to a generally stronger membrane or to a slower capacity to release chromium during the time of incubation Shown in Fig. 3C is the behavior of the two lines in a comparison between spontaneous and antibody-dependent cellular cytolysis Again, both parent and variant are killed equally well by the effectors of ADCC Thus, the NK-resistant cells can be killed by cellular mechanisms if the effectors are armed appropriately. We conclude that the variant is resistant on the basis of a mechanism other than refractoriness to a cellular lytic mechanism

Competition between Susceptible and Resistant Lines

Studies comparing the capacity of parent K562 and Clone I to inhibit the lysis of the other in the cold cell competition assay is shown in Fig 4 Figure 4A shows that while Raji does not inhibit the lysis of ^{51}Cr-labeled K562, Clone I does so in a manner intermediate to that observed for K562 against itself In contrast, when the targets are ^{51}Cr-labeled Clone I, there is slight inhibition by Raji but equal inhibition by itself or parent K562. We conclude that the resistance to lysis of Clone I is based upon a surface difference that renders it unrecognizable by the major population of unstimulated peripheral blood NK cells

Lysis by Activated Effector Cells

Interferon activates NK cells through possible mechanisms involving maturation from precursors, augmentation of target binding, or increased efficiency of lysis (20, 21) Staphylococcal protein A also has been reported to activate through the stimulation of interferon secretion (18) Shown in Fig 5 is the activity against Clone I of

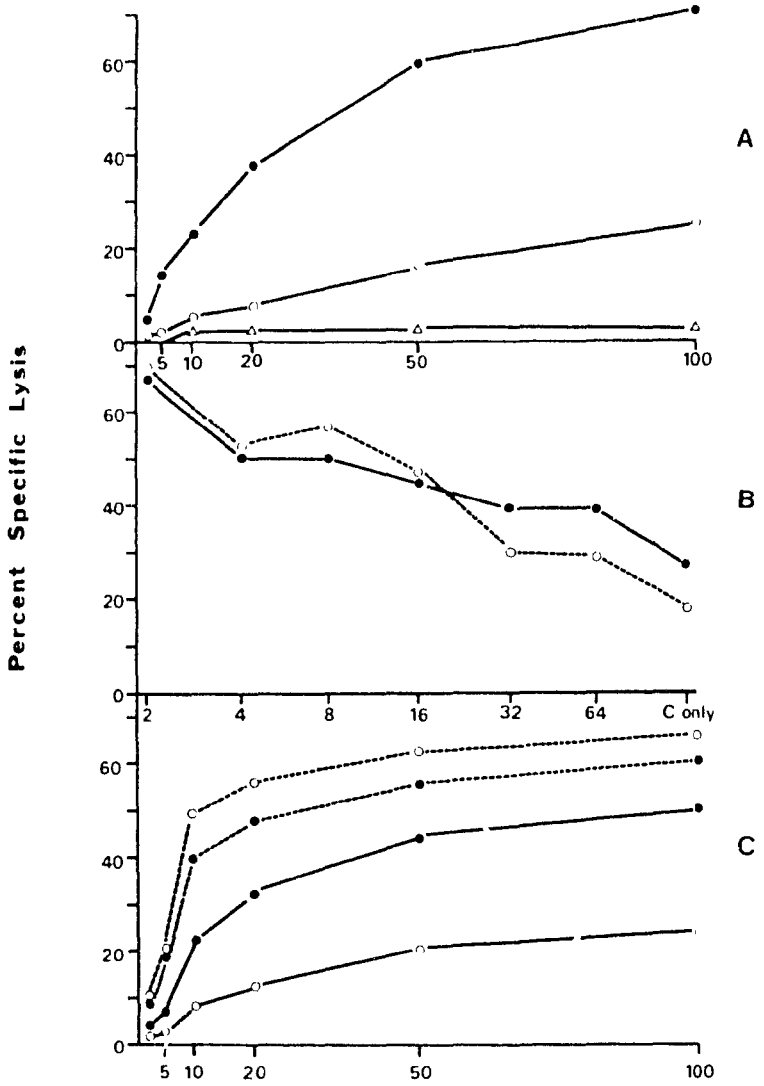

FIG 3 (A) Lysis of target lines as a function of effector cell number in the standard cytotoxicity assay shown for comparison Solid dots, parent K562, open dots, Clone I, triangles, Raji (B) Lysis of target lines as a function of dilution of a rabbit anti-K562 antiserum in the presence of complement Solid dots, parent K562, open dots, Clone I (C) Lysis of target cells as a function of effector cells in the ADCC assay Solid lines, untreated target cells, dashed lines, cells pretreated with a 1 4 dilution of the rabbit anti-K562 antibody Solid dots, parent K562, open dots, Clone I

lymphoid cells that had been incubated for 12 hr in the presence of protein A These data indicate that, although the lysis of the parent K562 is slightly increased, that of the variant is significantly augmented to within the range of the parent Thus, Clone I can be killed by effector cells whose efficiency or number have been increased by the activation process

DISCUSSION

These data show that resistant variants of NK-sensitive human leukemic lines can be selected without prior exposure to mutagen by repeatedly depleting effector–target

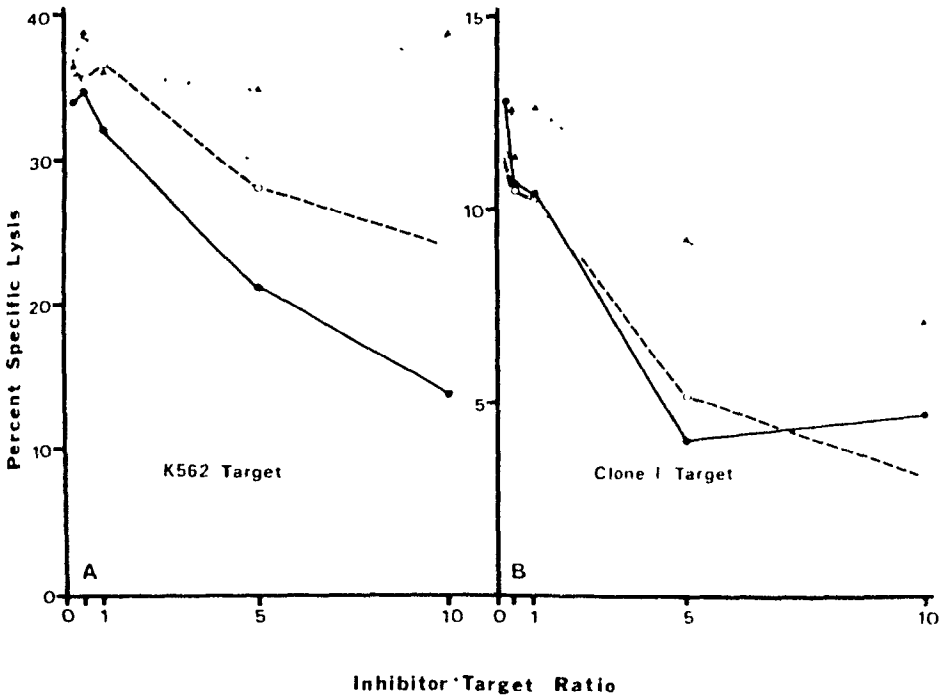

FIG 4 Cold inhibition of labeled targets expressed as a function of kill in the presence of the noted ratio of inhibitors to target cells (A) ^{51}Cr-labeled parent K562 (B) ^{51}Cr-labeled Clone I Inhibitor cells Solid dots unlabeled parent K562, open dots, unlabeled Clone I, triangles, unlabeled Raji

cell conjugates and cloning Furthermore, the new lines that grow maintain the resistant phenotype indefinitely Since these variant cells can be killed at a level equal to that of the parent by complement, antibody-dependent cellular cytotoxicity, and by protein A-activated effectors, the resistance occurs through mechanisms other than a general refractoriness to cytolysis Because of the close similarity of NK and K cells, it is probable that the mediators of lysis are the same in both Thus, we must seek an explanation for resistance at the level of stimulation of the toxic reaction The experiments on the effect of cold cell competition shown in Fig 4 suggest that the lesion is in a cell surface target structure This would be consistent with the claims of others that recognition and binding of the NK cell to its preprogrammed target is a necessary first step in the activation of the lytic mechanism (8) However, these studies do not enable a differentiation between the possibilities of an absent versus a masked binding site

There have been previous claims to the derivation of NK-resistant lines of mouse cells (9–12) but, to our knowledge, this is the first description of such a system in man. A comparison of their characteristics with the K562 lines described here point to potentially instructive differences. First, at some point all of the murine lines were subjected to *in vivo* passage where selective pressures may operate other than those caused by endogenous NK cells In fact, some of them revert to the sensitive state when returned to *in vitro* culture (9) Moreover, neither group published cytogenetic data to verify that their derivatives truly were derived from the alleged parent and not from a tumor arising *de novo* in the host animal during passage Indeed, Durdik

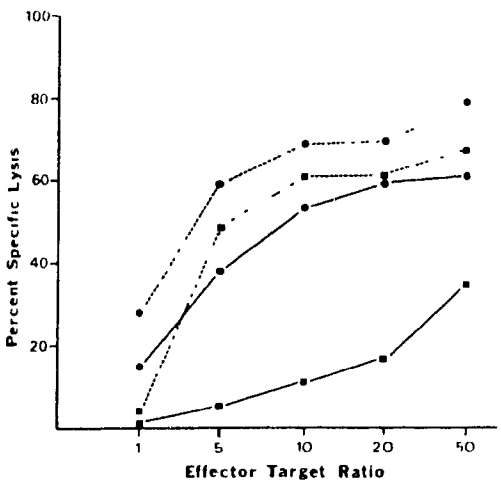

FIG. 5 Action of protein A-treated effector cells upon target lines Dashed line, cells preincubated for 12 hr with 50 μg/ml protein A Solid line, cells without protein A treatment Solid dots, parent K562 Solid squares, Clone I

et al (10) noted size and serologic differences that might lead one to suspect that possibility On the other hand, the K562 clones described here evolved totally through *in vitro* selection techiques based upon the functional parameter of binding and maintained surface antigenic and karyotypic markers of the parent Conversely, the report by Herberman (22) of unsuccessful attempts to obtain resistant K562 lines by cloning alone may have been due to the lack of statistical advantage enabled by preselection

The resistant murine lymphoma line of Durdik *et al* (10), designated a cl 27av, was observed to be totally refractory to NK action This complete resistance was interpreted, with their other studies (23), to support the hypothesis that the general capacity of various lines to compete against each other was due to a unispecific effector population and not to subgroups with different target-directed specificities This contrasts with our observation that Clone I is partially sensitive in relation to the more absolute resistance of the B-lymphoblastoid line Raji Since our initial negative selection procedure was based upon interaction of sensitive targets with a relatively large number of lymphoid cells with high affinity receptors for binding, it is possible that we have isolated K562 cells with either decreased, absent, or low affinity target structures for the quantitatively predominant NK-cell population We consider low affinity to be unlikely for, were a single NK-cell population attacking such a target, one would expect Clone I to compete against itself to a lesser degree than would the parent This is not what was observed in the experiment illustrated in Fig 4B Furthermore, the failure of prolonged incubation of 12–16 hr to narrow the kill ratio between the components of the cell pair makes untenable an explanation based upon kinetic considerations alone Assuming that multiple accumulative mutations did not occur in these clones, we find our data to be most consistent with the hypothesis illustrated in Fig 6 In this model, Clone I lacks an effective target structure present on its parent that is recognized by a majority NK population in unstimulated peripheral blood, but retains another common site recognized by a minority subpop-

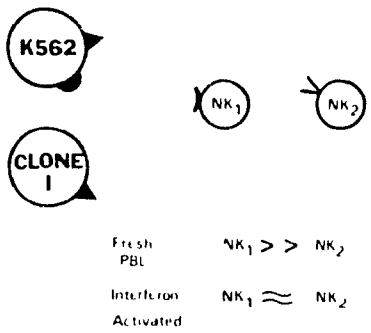

FIG. 6. Graphic representation of target and NK-receptor structures consistent with the results presented here. Triangles and half-circles on the K562 cells represent target structures; the reciprocal symbols on the NK cells represent their complementary receptors. For further explanation, see text.

ulation. Within the limits of sensitivity of the techniques used in the conventional competition studies and in those of Durdik *et al.* (23), the effect of such a minor population may be masked as long as cells bearing the major target were present.

In summary, our present interpretation proposes that the parent K562 may bear two target structures, one of which is recognized by a predominant and one by a lesser subpopulation of fresh human peripheral blood NK cells. In contrast, Clone I is equally sensitive to lysis but appears not to be recognized by the major group. Interferon stimulation of lymphoid cells could increase the number and/or activity of both effector types to a level where there would be maximal killing for both parent K562 and Clone I. These studies do not negate the possibility that other types of cells exist with totally different or polymorphic targets that are recognized by the same or other NK cells that bear the complementary receptors. We anticipate that experiments observing the effects of purified and cloned NK cells upon similar derivatives of other cell lines and its modulation by interferon will define these structures further.

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Publication 2: Target Cell Specificity of Human Natural Killer (NK) Cells II. Apparent Change with Activation

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The following statement regarding experimental contribution and authorship is made in accordance with the "Guidelines Concerning Thesis Preparation, Section 7: Manuscripts and Authorship", Faculty of Graduate Studies and Research:

The ideas, experiments, and writing represented in **Publication 2** were produced by Stephen L. MacDougall, with the supervision and input of Arthur K. Sullivan as thesis supervisor. Chaim Shustik performed a series of cold target competition experiments with protein A-activated effector cells that included the data presented in Figure 3, and was involved in the interpretation of this data.

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Target Cell Specificity of Human Natural Killer Cells

II. Apparent Change with Activation

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The activity of natural killer (NK) cells can be augmented by incubation with interferons, or with other compounds, such as staphylococcal protein A, which stimulate interferon production. In the experiments described here we compared the patterns of lytic activity of human lymphoid effector cells before (NK-B) and after (NK-A) short-term activation. Target cells used were K562, Clone I (a partially NK-resistant K562 variant), and those that had been preincubated with neuraminidase or trypsin. The results obtained include the following: (1) proteases but not neuraminidase, decreased lysis by NK-B but not by NK-A, of both K562 and Clone I in the standard Cr-release assay. (2) In the single cell assay, trypsin minimally decreased conjugate formation and the fraction of bound cells that were killed by NK-B, but did not reverse the increased lytic efficiency of NK-A. (3) In the cold-target competition assay, Clone I, which does not compete as well with K562 for NK-B, did so equally well for NK-A. (4) Trypsinized targets, regardless of their equal sensitivity to lysis by NK-A, were not as active competitors for NK-A. We conclude that the most reasonable interpretation is that K562 cells bear surface structures which can induce release of lytic mediators from NK-A under conditions that are not sufficient to stimulate NK-B. Although it appears that NK-A may respond to a smaller number of the same target molecules recognized by NK-B, the process must be better defined at the molecular level to exclude the possibility that there are qualitative differences between the proposed recognition structures for these two states of NK activity.

INTRODUCTION

Lymphoid cells that lyse susceptible targets *in vitro* without deliberate prior exposure to an antigen have been called natural killers (NK) (1). This activity is not indiscriminate but shows specificity in the types of cells that can be attacked (2). Although recognition and binding are thought to be an essential first step (3), the molecular nature of the involved ligands and receptors are largely unknown (4, 5).

It has been observed that NK activity can be increased by the immunoregulators interleukin 2 (IL-2) (6) and interferons (7, 8), which may operate through interconnected pathways (6–9). Other compounds, such as staphylococcal protein A (10, 11), stimulate interferon production. A consequence of this augmentation *in vitro* is an expanded range of vulnerable cell lines and a greater number of cells killed at a faster rate. Experiments using the single cell cytotoxicity assay have shown that contributing factors include recruitment of nonfunctional "pre-NK" cells as well as increased effector cells recycling (12–15). Although these observations account in part for the

greater degree of target lysis, they do not explain how the extended specificity occurs. Given the potential role of the interferons and IL-2 as tumor and immunotherapeutic agents, it is important to understand how activated cells behave toward targets that are less sensitive to unstimulated NK.

Since multiple subpopulations of NK cells may exist (16) and each potential target may possess more than one recognition site (17), further simplification could help to dissect such a multicomponent system. Even when two different cell lines of the same lineage are compared, many uncontrolled variables remain. For this reason we developed variants of K562 which differed in their interaction with NK. The line called Clone I was selected for decreased lysis by untreated PBL and showed equal sensitivity to antibody-dependent cytotoxicity and to protein A-activated effectors (18). In this paper we describe further studies on these cells using augmented NK and compare them to the behavior of protease-treated targets. Some of these results have been reported in a preliminary communication (19).

MATERIALS AND METHODS

All reagents were obtained from routine commercial sources except for leukocyte-derived recombinant interferon-α which was a gift from Dr. D. Parkinson (Tufts University, Boston, Mass.). Cell culture was by standard methods. The derivation and characteristics of the NK-resistant K562 subline Clone I, and the cytotoxicity and competition assays have been described elsewhere (18). Exposure of NK cells to staphylococcal protein A (Pharmacia) was according to the method of Catalona *et al.* (11) as used previously. Activation by interferon was carried out by incubation of PBL from healthy donors for 12–18 hr in RPMI 1640 media supplemented with 10% FCS and 1000 U/ml of interferon. All of the representative graphs shown are the result of at least three reproducible experiments.

Enzyme treatment of target cells. Target cells were labeled with ^{51}Cr as described previously (18) and were resuspended in 2 ml PBS containing either 1 mg/ml trypsin (TPCK-treated, Worthington), 5 mg/ml chymotrypsin (49.2 U/mg, Worthington), or 0.1 U/ml neuraminidase (protease free from *Vibrio cholerae*, Calbiochem). After 10 min of incubation at room temperature the enzyme reactions were terminated by dilution of the mixture to 12 ml with RPMI 1640/10% FCS when chymotrypsin or neuraminidase was used, or by the addition of 2.5 mg/ml soybean trypsin inhibitor (Millipore) when trypsin was used. Cells were washed three times in complete media and then checked for viability by trypan blue exclusion, which routinely was over 95%. Control targets were incubated in enzyme-free buffer and washed in a fashion identical to the treated cells. The NK assays with these cells were as described (18) but were shortened to 3 hr to minimize effects of membrane repair.

Preparation of LGL fraction. Large granular lymphocytes (LGL) were obtained by following a combination of described methods (20, 21) including Percoll (Pharmacia) gradient fractionation of peripheral blood lymphocytes preseparated on a Ficoll–Paque (Pharmacia) barrier, and depleted of adherent cells on a nylon wool column (22). Briefly, $1–2 \times 10^7$ cells in 0.5–1.0 ml from the column were loaded onto a discontinuous Percoll gradient in 15-ml conical polystyrene tubes. The gradient consisted of six steps in 2.5% increments from 42.5–55% (v/v) over a 0.5-ml 100% cushion, 100% was defined as nine parts Percoll and one part 10× concentrated PBS to maintain isotonicity. The tubes were centrifuged at room temperature at 500*g* for

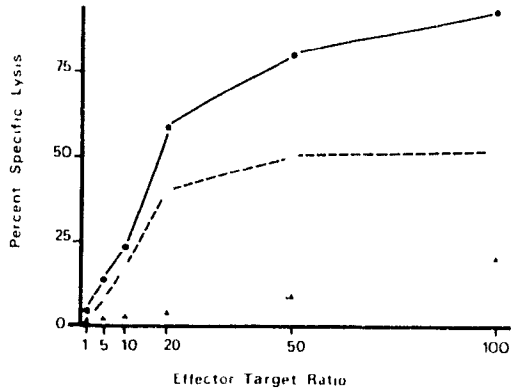

FIG. 1. Natural cytotoxicity against cell lines by effector cells incubated with targets for 18 hr without prior exposure to protein A or interferon. Solid dots, K562; open dots, Clone I; triangles, Raji.

30 min. The highest NK activity was found to be at barriers 3–4 and 4–5, and the cells with little activity, the majority, were at barrier 5–6. In all experiments using LGL the cells at the 3–4 and 4–5 interfaces were pooled, washed three times in 1640/10% FCS medium, and used for further experiments.

Single cell assay. Determination of conjugate-forming cells and lytic conjugates was as described by Bradley and Bonavida with minor modifications (23). Briefly, 0.1 ml of effector cells (fractionated as large granular lymphocytes) at 5×10^6/ml was mixed with 0.1 ml of target cells at 2×10^6/ml in 10 × 75-mm polystyrene culture tubes, centrifuged for 5 min at 250*g*, and incubated for 30 min at 37°C. After incubation the supernatant was removed, 50 μl of RPMI 1640/10% FCS was added, and the pellet was gently resuspended by mixing four times with a 50-μl Eppendorf micropipette. Agar, 50 μl at 37°C, was added and the mixture was layered onto glass slides for incubation and subsequent enumeration.

RESULTS

Competition between K562 and Clone I for Activated Effector Cells

Previous studies have shown that the K562 variant, Clone I, was not killed to the same degree as the parent by fresh peripheral blood lymphoid cells and did not compete as effectively in the cold target inhibition assay (18). To eliminate the possibility that the different sensitivity to lysis between K562 and Clone I was merely due to different rates of killing, the cytotoxic assay was prolonged from 4 to 18 hr. The results in Fig. 1 indicate that although the overall killing of both lines was increased, the difference between the two lines remained. In contrast to this pattern it was found that if the effector cells were incubated in the presence of protein A for 12–18 hr before assay, the two target cells were killed equally well. The experiments shown in Figs. 2 and 7 extend these observations to show that in addition to being as sensitive to lysis, Clone I became a potent competitor of K562. The observations that Raji cells remained both a weaker target and competitor indicated that some selectivity of killing was retained. These findings on the behavior of protein A-activated NK have been confirmed using recombinant interferon α (data not shown).

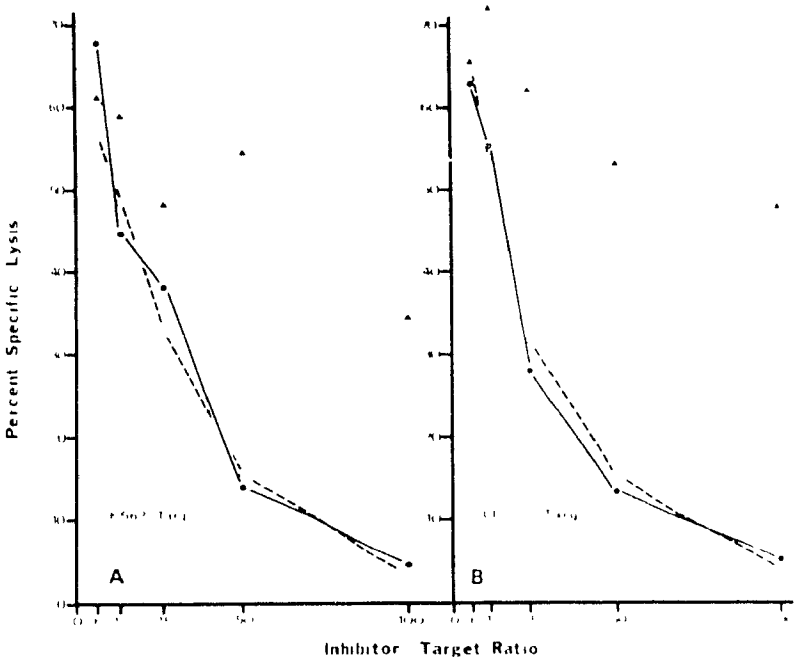

FIG. 2. Cold target competition assays with protein A-activated effectors. Solid dots, K562 unlabeled competitor; open dots, Clone I; solid triangle, Raji. A, K562 labeled target. B, Clone I labeled target.

Competition between Other Lines for Activated Effectors

Subpopulations might exist within the resting NK pool which could have different spectra of target discrimination and different responses to activators (16). Relative changes in their proportions could have led to the above observations. To assess this further, the ability of the K562 lines to compete was compared to that of a lymphoid-derived target, CCRF-CEM, selected for intermediate sensitivity to lysis by untreated effectors (i.e., between that of K562 and that of Clone I) but of equal sensitivity to lysis by augmented NK. In these experiments with activated NK (Fig. 3) both K562 and Clone I competed equally well with autologous targets and with CEM; on the other hand, CEM was not as potent a competitor. Were there simply a relative shift in the proportion of effector subpopulations with different specificities, one could have anticipated that the increase in killing of CEM would have been reflected also in its capacity to compete against itself. These results confirm that the assay can reveal a degree of inhibition greater than that of an autologous combination (i.e., CEM against CEM), and that the ability to compete does not necessarily follow from what might be predicted from the pattern of sensitivity to lysis (24).

Effects of Neuraminidase

It has been reported that the quantity of neuraminidase-releasable sialic acid correlates inversely with effector–target binding and subsequent lysis (25). To eliminate the possibility that such structures may contribute to the differences between the two

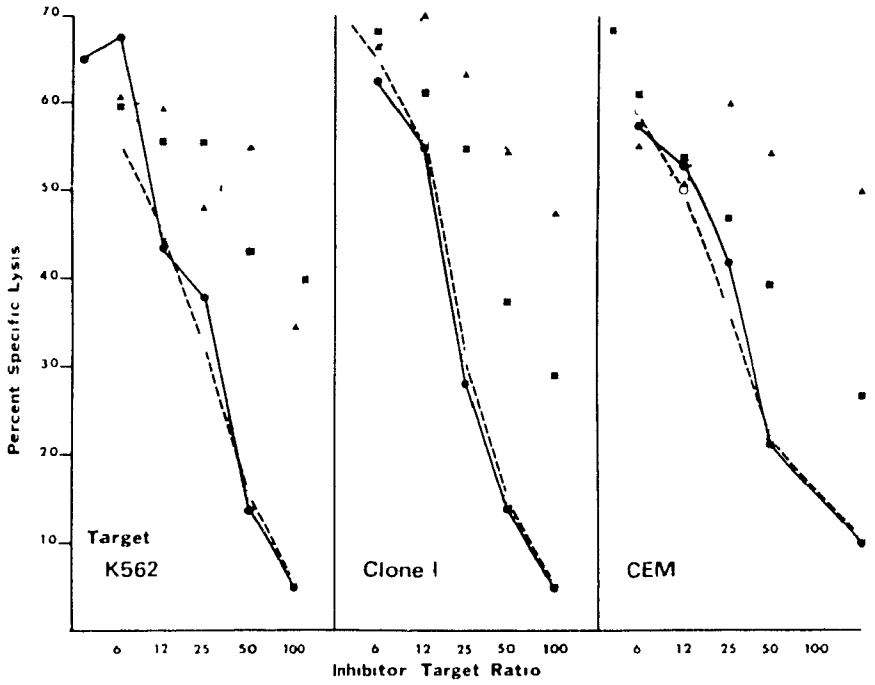

FIG. 3. Cold target competition assays with protein A-activated effectors showing behavior of the CEM line. Solid dots, K562 unlabeled competitor; open dots, Clone I; solid triangles, Raji; solid square, CEM. The radiolabeled target cell is indicated at the bottom of each panel.

K562 lines we studied the effect of this enzyme on lysis by both basal and augmented effector cells (Fig. 4). Neuraminidase treatment resulted in a slight increase in kill of both lines, but it did not change their relative sensitivities to either basal or activated NK. This indicates that changes other than those involving enzyme-sensitive sialic acid and the effects of their negative charges on intercellular distance must be responsible for the patterns observed with Clone I targets.

Effects of Proteolytic Enzymes

Cell surface target structures for NK recognition are sensitive to certain proteases (26, 27). Exposure of K562 and Clone I to either trypsin or chymotrypsin, under conditions sufficient to remove greater than 95% of the ethanol-precipitable ^{125}I counts incorporated by lactoperoxidase, resulted in diminished lysis of both lines by untreated effectors. However, K562 remained slightly more sensitive than Clone I. When the same enzyme-treated targets were exposed to activated cells, both lines were killed as well as the parent K562, which had not been subjected to external proteolysis (Figs. 5, 6). Thus, the surface structures needed to activate and respond to the lytic mechanisms of untreated effectors were rendered less potent by treatment with protease, whereas those involved with augmented NK remained sufficient.

If the capacity of protease-treated K562 and Clone I to initiate killing by augmented effectors were mediated through a set of target structures different from those

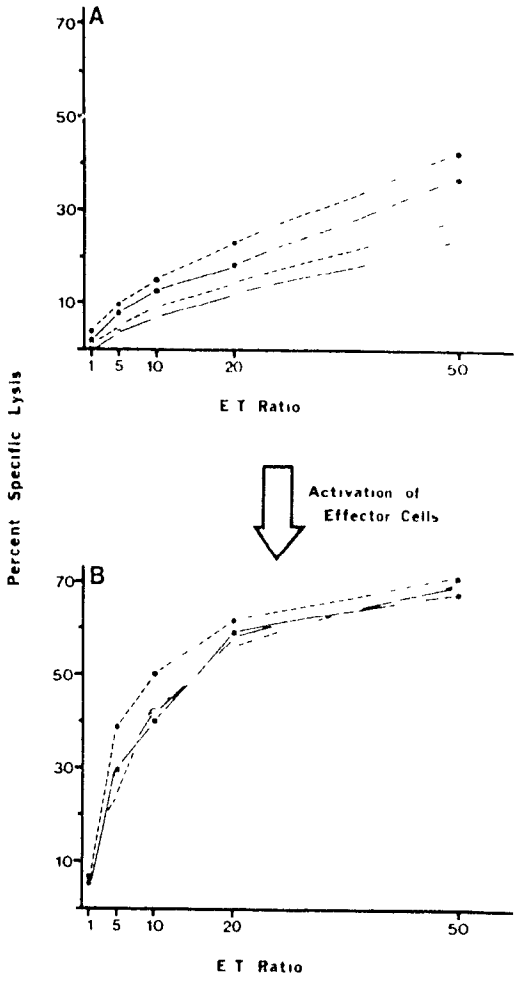

FIG. 4. Effect of neuraminidase on targets assayed in a 3-hr NK assay with untreated and activated effectors. Designation of cell lines as in Fig. 1. Solid lines, untreated targets; dashed lines, enzyme-treated targets. A, untreated effectors; B, protein A-treated effectors.

recognized by basal NK cells, the new pattern of competition should be similar for all four states of K562 (i.e., K562 and Clone I, treated and not treated with enzyme). When trypsin-treated cells were assessed for their capacity to retard lysis of K562 by protein A-treated effectors in the cold-target assay, the patterns shown in Fig. 7 were obtained. In neither combination of labeled indicator cells were the trypsin-treated competitors as efficient as the untreated. Moreover, treated Clone I competed less against both the parent and itself than did treated K562, suggesting that there was a dissociation between the quantity of membrane components needed for initiating the lytic process and those required to diminish the accumulated binding events measured by competition.

Behavior in the Single Cell Assay

Exposure of NK cells to interferon has been shown to increase their rate of recycling and, in some undefined manner, to increase the efficiency of killing. Conceivably, the

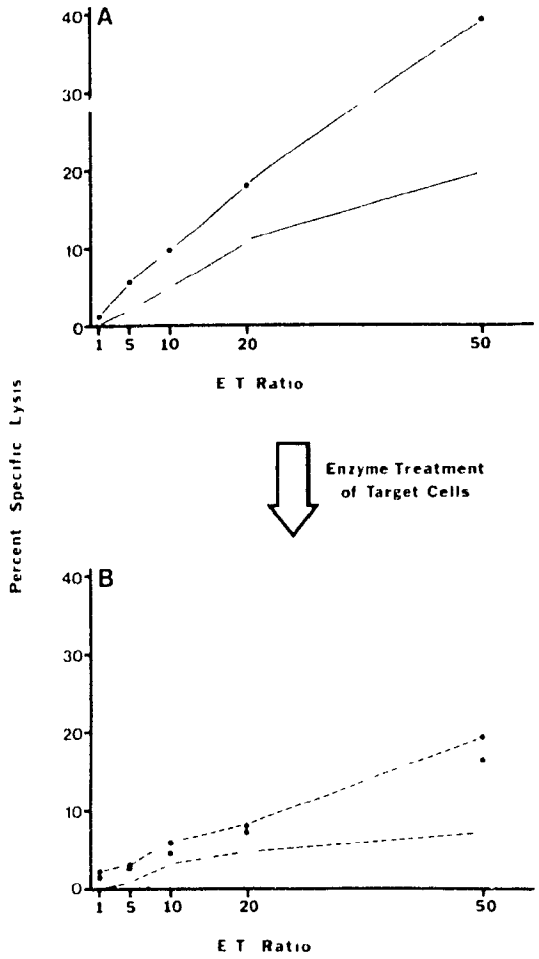

FIG. 5. Effect of proteases on targets assayed with *untreated effectors* in a 3-hr assay. Designation of cell lines as in Fig. 1. A, untreated targets; B, protease-treated targets. Dashed line, trypsin treatment; dotted line, chymotrypsin treatment. Solid dots, K562; open dots, Clone I.

marked increase in kill of Clone I and the protease-treated targets could have been due to augmented effectors having a more rapid rate of lysis and recycling. The single cell assay measures NK activity of target/effector conjugates immobilized in a semi-solid matrix where recycling can not occur. The results of several such assays, summarized in Fig. 8, show the patterns of binding and killing after activation of effectors, or trypsin treatment of targets. The changes in binding after activation of effectors or protease treatment of targets were minimal. More definite trends were observed in the capacity of bound cells to kill. Although killing efficiency by basal NK cells was decreased by trypsin, the increased activity of augmented effectors was not

DISCUSSION

Using two manipulations of K562 cells that decreased their sensitivity to lysis by unstimulated NK (i.e., selection of variants and protease treatment), we have shown

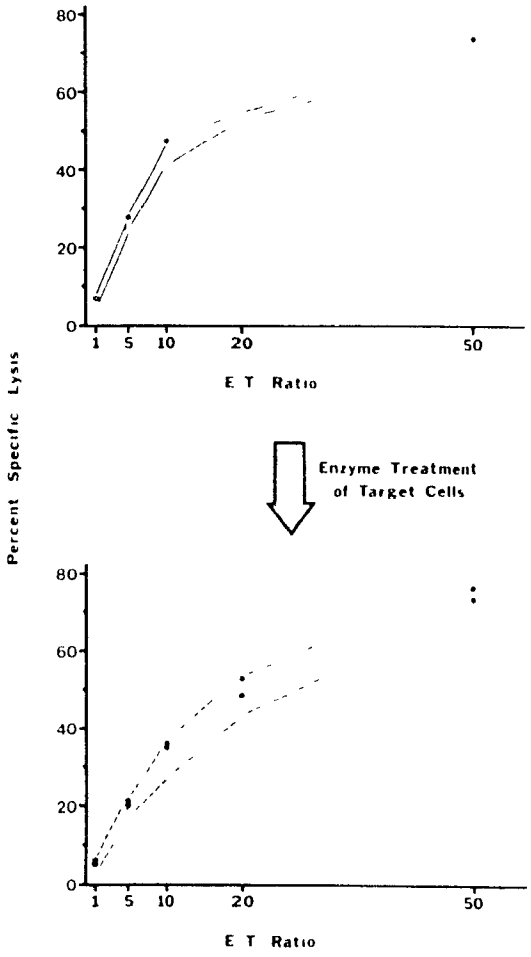

FIG. 6. Effect of proteases on target cells assayed *with protein A activated effectors*. Graphic designation is for Fig. 5.

that such targets can be killed in a ^{51}Cr-release assay by effectors that have been activated by short-term incubation with protein A. This might occur if NK-A(activated) and NK-B(basal) could recognize different target structures or if NK-A could respond to a less potent stimulus. The single cell assays indicated that there was not a great difference in the quantity of binding of either effector cell to Clone I or trypsinized K562. Although trypsin decreased the percentage of bound K562 that were killed by NK-B, it did not diminish killing by NK-A. This could imply, within the sensitivity of this technique, that the effects occurred after the binding step. Results of the competition assays suggested that the surface structures recognized by NK-A were similar on parent K562 and Clone I, but those on protease-treated targets were fewer in number. Since all of this inference is subject to alternate interpretation and should not be extended beyond the limitations of the complex assays involved, these considerations will be discussed below within the framework of current models for NK action.

It has been proposed that a complex sequence of events leads from initial recognition of a target by an NK cell, to transfer of a "lytic complex" to the target cell surface

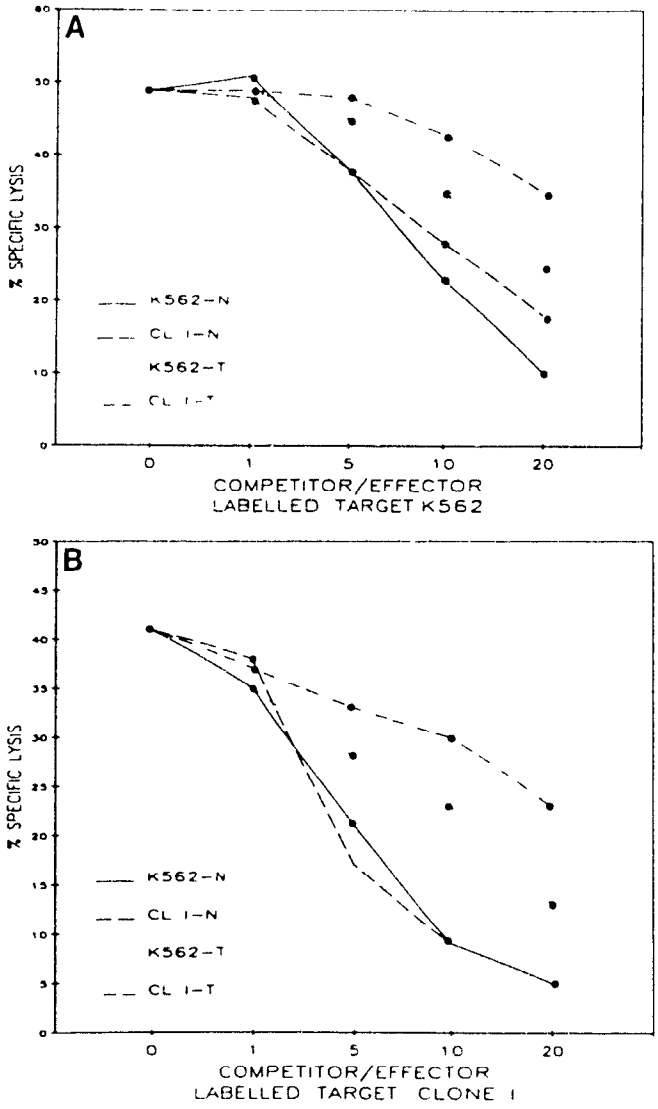

FIG. 7. Cold target competition of augmented effector cells. Capacity of nontrypsinized (NT) and trypsinized (T) cells to compete with ^{51}Cr-labeled K562 (A) or Clone 1 (B) targets for lysis by augmented effectors. Representative curve of three consistent experiments.

and finally to local release of cytotoxic factor (NKCF) which must bind to a receptor to effect its damage to the membrane (27). The recognition site and "lytic complex" were sensitive to the proteases trypsin and chymotrypsin, but the putative receptor for cytotoxic factor was not (27). Other studies have implicated proteases as well as complement-like components at intermediate steps in lysis (28, 29). Although most of those studies used untreated lymphoid cells, experiments on Sr^{2+} inhibition of effector degranulation and killing indicate that the toxin secretory mechanisms of both interferon-exposed and basal effectors possess common features (20, 30) consistent with the granule exocytosis model (31). Also, Wright and Bonavida (32) have

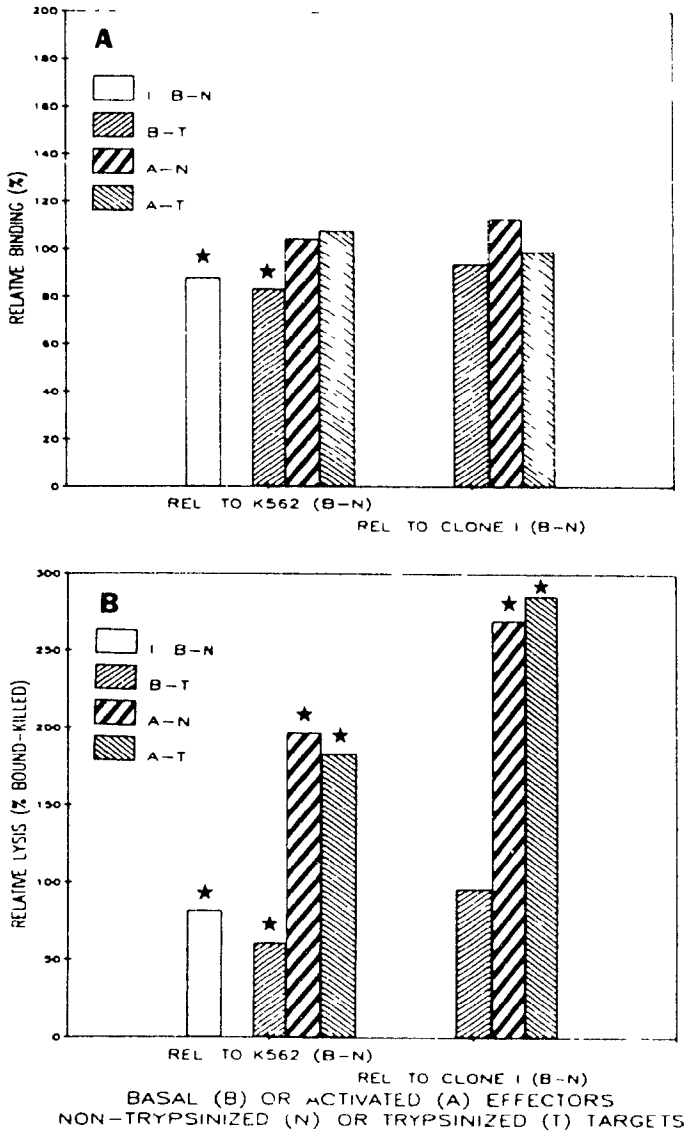

FIG. 8. Effects of activation of LGL on lysis of trypsinized (T) and nontrypsinized (N) targets in the single cell assay. A, conjugate formation. B, percentage of bound conjugates in which the target was killed. In order to compare the results between separate experiments, the value obtained for each determination was expressed as a percentage of the value measured for the untreated (N) target exposed to unactivated (B) effectors. This baseline value was arbitrarily assigned 100%, thus the height of each bar reflects the change from baseline that resulted from the described manipulations. The left sides of A and B show the results obtained with K562 compared to the baseline 100% value defined above, the right sides show the results obtained with Clone I compared to the Clone I 100% baseline. For comparison, the value obtained for untreated Clone I relative to the K562 baseline is shown by the open bar (I B-N) at the beginning of each panel. The stars indicate the effector/target combinations for which the means obtained from six independent experiments for each bar, are different from 100% at the $P < 0.05$ level as determined by the standard one-tailed t test.

found that the NKCF activity increases in cultures that contain interferon. Thus, it appears that the final lethal molecules may be the same for both basal and activated killing

It has been shown that NK cell activity can be augmented so that known targets are killed more vigorously, and resistant lines become sensitive Mechanisms proposed to explain these findings have included the recruitment of inactive cells, increased rate of killing, and increased recycling (13, 14) Were the greater kill of trypsinized K562 and Clone I by activated NK simply due to a greater rate of recycling, a longer time of exposure to unactivated cells should have brought it closer to that of the original line, which it did not Neither would one have expected the pattern of competition to have changed after effector cell stimulation as it did Furthermore, the increase in killing of trypsinized targets occurred also in the single cell assay in which recycling was prohibited

To explain why the lysis of trypsinized cells by basal NK decreased and that by augmented NK remained intact, the following two possibilities must be considered First, the target structures recognized by activated effector cells might be different, as well as protease resistant Alternatively, activated cells might be stimulated to release their lytic mediators after interaction with fewer of the same target structures Such suggestions are compatible with some of the known effects of interferon, such as stimulation of cells to produce new cytoplasmic and surface proteins, in a time period similar to that of the present studies (33–35) However, an independent receptor-ligand system leaves unexplained why the protease-treated cells did not compete in proportion to their kill The second possibility might occur through a more sensitive coupling of stimulus to secretion of the lytic apparatus, such as might result through changes in membrane fluidity (36, 37) Since interferon has been shown to stimulate release of measurable NKCF (32), and the secretion of NKCF and interferon-γ to be under similar control (38), further experiments could be designed to determine if the quantitative requirements of target cells to stimulate NKCF are different in basal and interferon-treated effectors

Were the lytic process of cell-mediated cytotoxicity analogous to that followed by complement, as has been proposed, one might expect that such a cascade, initiated by contact and consequently amplified, would include sites where alternate pathways could interact In such systems the early steps with the greatest specificity serve to focus dilute ambient activities (e g , complement or coagulation factors) to where there is immediate need, the later alternate pathways are less discriminating Many characteristics of the NK response to interferon appear to follow this paradigm early specific recognition of target by effector, stimulation of interferon-γ production by NK cells, and augmentation of lytic activity by several mechanisms, including one in which stimulation and action of the final lytic complex may short circuit the usual route Indeed, Rosse and co-workers have shown in another system that trypsin treatment of erythrocytes leads to increased C3 binding and activation, and that the aberrant erythrocyte plasma membranes of some patients with paroxysmal nocturnal hemoglobinuria can passively absorb C5b-9 from the fluid phase (39) without prerequisite antibody binding From the experiments described here, we can not exclude the possibility that augmented NK cells kill trypsinized K562 and Clone I by an alternate pathway, such as one which might circumvent the trypsin-sensitive components of the "lytic complex" (27) However, to explain the results of the competition assays, we would yet have to implicate a further change at the recognition level

The NK phenomenon has been studied by several different techniques which may lead to conflicting interpretations Many extrapolations have been made from studies of lysis, binding, and cold-target competition, but there is little known at the molecular level of the requirements necessary for each activity For example, although it has been reported that "NK-patterned binding" without lysis may occur in a panel of cell lines (24), the specificity and affinity of the surface molecules involved may be sufficiently different to give divergent results among the three methods Because of the greater number of cells involved, competition may be more sensitive, and more accurately reflect accumulated interactions between potential target and effector cells than do the somewhat subjective single cell binding assays Such a distinction could explain why the ability of Clone I to compete with basal NK correlated more closely with its decreased sensitivity to lysis than did its tendency to form conjugates with lymphoid cells in the single cell assay When the potency of effector cells, measured in the standard Cr-release assay, was increased by activation, and the sensitivity of target cells was decreased by protease, the differences between kill, binding, and competition became more evident At this end of the spectrum, trypsinized targets formed conjugates and were killed as efficiently as the untreated, but they were not able to compete to the same degree Alternatively, it is possible that the proteases might have decreased the number of specific binding sites, but concurrently increased the general stickiness of the surface All of these possibilities will remain speculative until the relevant cell surface molecules have been identified and characterized, and it can be shown with precision what and how many are required for binding, what interactions are necessary for effector stimulation, and what are the quantitative relationships between binding, competition, and killing

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Publication 3: Increased fucosylation of glycolipids in a human leukemia cell line (K562-Clone I) with decreased sensitivity to NK-mediated lysis

S. L. MacDougall, G. A. Schwarting, D. Parkinson and A. K. Sullivan

Immunology 62: 75-80 (1987).

The following statement regarding experimental contribution and authorship is made in accordance with the "Guidelines Concerning Thesis Preparation, Section 7: Manuscripts and Authorship", Faculty of Graduate Studies and Research:

The ideas, experiments, and writing represented in **Publication 3** were produced by Stephen L. MacDougall, with the supervision and input of Arthur K. Sullivan as thesis supervisor. G. A. Schwarting and D. Parkinson consented to perform the glycolipid analysis of the cell lines, and participated in the interpretation of this data (Figure 3 and Table 1).

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Increased fucosylation of glycolipids in a human leukaemia cell line (K562-Clone 1) with decreased sensitivity to NK-mediated lysis

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SUMMARY

A subpopulation of human lymphoid cells called natural killers is able to lyse certain normal and neoplastic targets in an *in vitro* cytotoxicity assay. The molecules which enable them to recognize sensitive cells, or permit tumour cells to escape remain unknown. In the studies described here we have compared some of the plasma membrane characteristics of a NK-sensitive human leukaemia cell line (K562) with those of a partially resistant subclone derived from it (K562-Clone 1). Gel electrophoresis of cell surface proteins radiolabelled by lactoperoxidase catalysed iodination, periodate borohydride tritiation or biosynthetically by incubation with [^{3}H]fucose did not reveal any reproducible differences between the sensitive and resistant lines. However, analysis of glycolipids showed that Clone 1 incorporated significantly more fucose than did the parental line, and that it synthesized a minor population of complex structures not found in the original K562. A subclone of Clone 1 (Clone 1 Con A$^{(+)}$) made resistant to the toxic effects of concanavalin A became sensitive once again to NK, and showed the parental glycolipid profile. These results suggest that the Clone 1 line selected for resistance to NK, may have altered one or more of its intermediate oligosaccharides or pathways of fucose incorporation into glycolipid, and points to one process by which a tumour cell might modulate its surface to escape recognition by natural killers.

INTRODUCTION

Natural killer cells, identified as a subpopulation of large granular lymphocytes, possess the capacity to lyse certain normal haemopoietic cells (Hansson, Kiessling & Andersson, 1981) and tumour cells (Herberman & Holden, 1978) *in vitro* without the prerequisite of deliberate host immunization. It has been proposed that they may perform a role in tumour surveillance *in vivo* (Warner & Dennert, 1982). For such a process to occur, susceptible cells must possess specific elements that enable their selective recognition from the main mass of normal tissues, and evoke the subsequent lytic events (Hiserodt, Britvan & Targan, 1982). The identity of such target molecules has been elusive. Although Roder *et al.* (1979) and Oberer, Rumpold & Kraft (1983) have claimed to have isolated possible target peptides, it remains uncertain if such partially denatured or altered products are those that mediate specific receptor/ligand recognition. Other workers have suggested that receptors for transferrin (Barnes, Lafleur & Holbein, 1983; Vodinelich *et al.*, 1983) might enable binding and activation of lysis.

Another approach has been to derive and analyse cultured cell lines refractory to NK-mediated lysis. Young *et al.* (1981) and Yogeeswaran *et al.* (1981) have shown that cells of murine lines resistant to both unstimulated and activated NK lack asialo G_{M1}. Howe[illegible] they concluded that these glycolipids were not the targets themselves. Using human cells, we have derived a variant of the leukaemic line K562 which is partially-resistant to being killed by fresh peripheral blood lymphoid cells, but is sensitive to antibody and complement, ADCC and interferon activated effectors (MacDougall, Shustik & Sullivan, 1983). Since this subline was selected primarily for inefficient binding, and competed less well with the parent K562 in the cold target assay, we proposed that it might bear decreased or deficient target structures, and be less competent in activating the lytic mechanism of the major population of unaugmented NK cells. This paper describes an initial biochemical comparison of the surface components of these NK-sensitive and resistant K562 lines.

Abbreviations: ADCC, antibody-dependent cellular cytotoxicity; AG_{M1}, asialo G_{M1}; CDH, ceramide dihexoside, CMH, ceramide monohexoside; Con A, concanavalin A; CTH, ceramide trihexoside; GLOB, globosides; SDS PAGE, sodium dodecyl sulphate polyacrylamide gel.

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MATERIALS AND METHODS

Manipulation of cells and derivation of cell lines

Cell culture methods and the derivation and characteristics of the NK resistant K562 subline Clone 1 have been described in detail elsewhere (MacDougall *et al.* 1983).

For flow cytometric analysis 2×10^6 cells were incubated for 30 min on ice with 0.5 ml of the diluted antibody containing ascitic fluid and after washing with 0.2 ml of the fluoresceinated second antiserum (goat anti-mouse IgG, Cappel Laboratories, Westchester, PA).

Derivation of cells resistant to growth inhibitory effects of concanavalin A (Con A) was by a step wise method in the absence of any added mutagens. Initially cells were grown at a concentration of 10^5/ml in 10 ml culture flasks in RPMI 1640 + 10% FCS medium with 25 μg/ml of Con A. Most of the cells died, but those which survived were expanded and recultured in media with 50 μg/ml Con A. These steps were repeated each time increasing the lectin concentration by 50 μg/ml until 200 μg/ml was reached. Cells then were grown in absence of Con A for 2 weeks, were re-exposed to 200 μg/ml and did not show any visual signs of toxicity. After this exposure/removal cycle was repeated four times, the cells were removed from the lectin for two passages and an aliquot was cultured at 10 cells/well in six well culture dishes containing 0.28% agar. When colonies became visible macroscopically, samples were picked randomly and expanded for further study. All clones were retested for sensitivity to Con A and remained fully viable after four passages in 200 μg/ml.

Analysis of cell proteins

Cells with a viability of greater than 95% by Trypan blue exclusion were used from a culture subdivided 2 days previously. For analysis of surface proteins, 10^7 cells were radioiodinated by lactoperoxidase catalysis and ethanol precipitation from a Triton X–100 extract, according to Walsh & Crumpton (1977) with modifications described by Peyman, Schwarting & Sullivan (1986). Detergent solubilization and lectin affinity chromatography were used according to published methods (Sullivan *et al.*, 1979) with the modification of a two-step elution from Sepharose-Con A (Pharmacia). The low affinity glycoproteins were eluted by 50 mM α-methyl mannoside and the more tightly bound components by 3 M KSCN. Tritiation of cell membrane glycoproteins was according to the procedure of Gahmberg & Andersson (1977). Gel electrophoresis was carried out by the method of Laemmli (1970). Samples containing equal quantities of radioactivity were added to each lane.

Biosynthetic labelling of glycoproteins

For fucose incorporation into glycoprotein, 150 μCi of L-fucose (5,6-^{3}H) (ICN Radiochemicals Canada Ltd, Montreal, Quebec), were added to 2.5×10^5 cells/ml in 10 ml of RPMI-1640/10% FCS medium and incubated overnight at 37°. After washing three times with PBS, stock Triton X–100 was added to make a 1% final concentration. Nuclei were sedimented, and particulate matter removed by centrifugation at 10^5 *g* for 60 min. For electrophoresis, proteins were precipitated in absolute ethanol at −20° overnight and removed by centrifugation.

Analysis of glycolipids

For analysis of lipids 5×10^6–6×10^6 cells were extracted in 50 ml of chloroform/methanol (2:1) for 5 hr, filtered, then re-extracted in chloroform/methanol (1:1) for 1 hr. The combined extracts were then dried, taken up in methanol and placed on a 5 cm × 5 mm column of DEAE Sephadex A-25. The neutral glycolipids were eluted with 30 ml of methanol and the gangliosides were eluted with 30 ml of 0.5 M ammonium acetate in methanol and desalted as previously described (Williams & McClure 1980). The neutral glycolipids were base treated to remove phospholipids, desalted and purified on silicic acid as described elsewhere (Schwarting & Summers, 1980).

For radiolabelling of glycolipids 10^7 cells were incubated for 18 hr with 100 μCi of either [^{3}H]fucose or [^{3}H]galactose, harvested and extracted as above. Thin layer chromatography plates containing radioactive glycolipids were exposed to Ultrafilm (LKB Bromma, Sweden) for 4–14 days at room temperature.

RESULTS

Major surface peptides

Lactoperoxidase catalyses the radioiodination of peripheral tyrosine-containing surface proteins that might be expected to have a role in intercellular interactions (Walsh & Crumpton 1977). Shown in Fig. 1a is a representative comparison of the major labelled polypeptides of parent K562 and Clone 1. Neither qualitative nor certain quantitative differences could be distinguished between the 28 major bands of the two lines. Thus the phenotypic difference in sensitivity to lysis by NK cells does not lie in gross changes in the membrane protein composition.

Carbohydrate-containing peptides

Roder has claimed that the target structures he has identified bind to Con A (Roder *et al.* 1979). As shown in Fig. 1b and c, in neither of the electrophoretic patterns of radioiodinated peptides that were eluted from Con A Sepharose by α-methyl mannoside or 3 M thiocyanate were any consistent differences noted between the sensitive and resistant cells. Since this method detects only a subpopulation of glycoproteins that have a high content of mannose, the more heavily sialylated structures were labelled by the periodate/borohydride technique and the major fucose-containing structures by biosynthetic incorporation of the tritiated monosaccharide. Again in none of more than 50 electrophoretic bands shown in Figs 1d or 2 were any major differences noted between the products of the two K562 lines.

Glycolipid structures

Although target cell sensitivity is susceptible to proteases (Pross, Luk & Baines 1978) implying a protein mediated process, alterations in the glycolipid environment may modulate the function of the binding sites (Bremer *et al.*, 1984, Muller & Shmitzky, 1979). To assess possible changes in the glycolipids, both the total lipid fraction and those biosynthetically labelled with radioactive monosaccharide were extracted and analysed by thin layer chromatography. The resulting profiles of K562 were very similar to what has been reported for this cell line

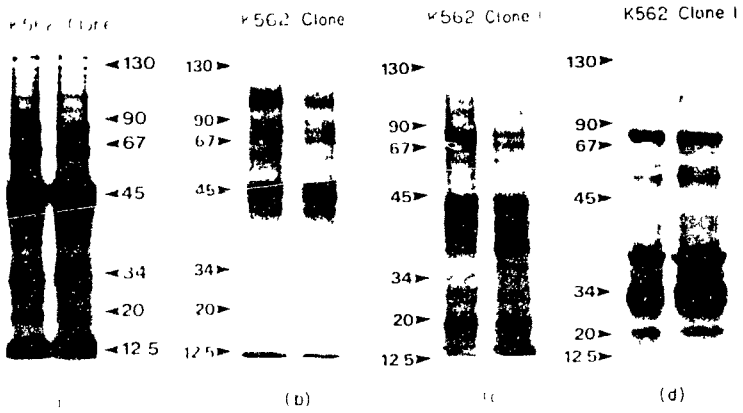

Figure 1. (a) Autoradiogram of SDS PAGE electrophoresis on 10% gels made of ethanol precipitated proteins of cells radioiodinated by the lactoperoxidase technique. The profile is representative of three independent experiments in which the intensity and position of the major bands were reproducible. Some minor bands showed differences in intensity in different experiments. (b) Autoradiogram of radioiodinated peptides eluted from Sepharose Con A by 50 mM α methyl mannoside. (c) Autoradiogram of radioiodinated peptides eluted from Sepharose Con A by 3 M KSCN. (d) Autoradiogram of ethanol precipitated glycopeptides labelled by the periodate tritiated borohydride technique.

Figure 2. Autoradiogram of 7% SDS PAGE electrophoresis of ethanol precipitated proteins of cells biosynthetically labelled by tritiated fucose.

elsewhere (Suzuki *et al.* 1981). In both lines the major neutral glycolipids had mobilities near those of CMH, CDH and paraglobosides (Fig. 3a), and the major gangliosides near those of G_{M3}, G_{M2}, G_{M1}, and G_{D1a} (Fig. 3b). However, Clone I possessed a group of glycolipids of low mobility that were not seen in parent K562 (Figs 3a and b). Consistent differences were not evident in the position for asialo G_{M2}, which was a relatively minor component. This was confirmed in the patterns obtained after incubation of cells with the radioactive monosaccharides galactose and fucose. Overall incorporation of galactose into both neutral glycolipids and gangliosides was the same for both lines, but that of fucose was many times greater in Clone I (Table 1). The chromatographic patterns shown in Fig. 3c demonstrate that tritiated fucose was distributed into similar components in both cell lines. However, Clone I contained a group of fucosylated structures that were not seen in the parent K562.

Expression of a major fucosylated surface antigen

To determine if the difference in fucose content was reflected in a major fucosylated trisaccharide cell surface antigen, the two K562 lines were compared for their capacity to bind a monoclonal antibody (H36-71, Peyman *et al.* 1986) reactive with the lactoneofucopentaose III determinant. This developmental antigen, also called X-determinant, stage-specific embryonic antigen, and My-1, can be borne on both lipid and protein, and consists of galactose in 1-4 and fucose in 1-3 linkage to N-acetylglucosamine (reviewed by Fenzi, 1985). Harris *et al.* (1984) have suggested that this structure may function as a target for NK cells. As shown in Fig. 4 of the profiles obtained from flow cytometric analysis of parent K562 and Clone I, both lines expressed essentially identical quantities of the antigen on their surface. Thus, the increased fucose incorporation observed in Clone I is not followed by a general increase in production of fucose-bearing glycolipids, and the new structures shown in Fig. 3 may represent a specific subpopulation.

Reversion of the resistant phenotype

Subclones of Clone I were selected for resistance to growth inhibition by Con A and then tested for sensitivity to NK lysis. Of 12 randomly selected subclones picked from agar and expanded in culture (described in the Materials and Methods), five were as sensitive to NK lysis as the parent K562, six were within 75%, and one remained resistant like Clone I. One of the five which had regained full sensitivity, designated Clone I Con A^{rev}, was selected for further study (Fig. 5). To determine if the altered pattern of fucose incorporation into glycolipid persisted in these 'revertant' cells, biosynthetic labelling studies were performed as described in the section above. In these experiments the degree of fucose incorporation (Table 1) as well as the

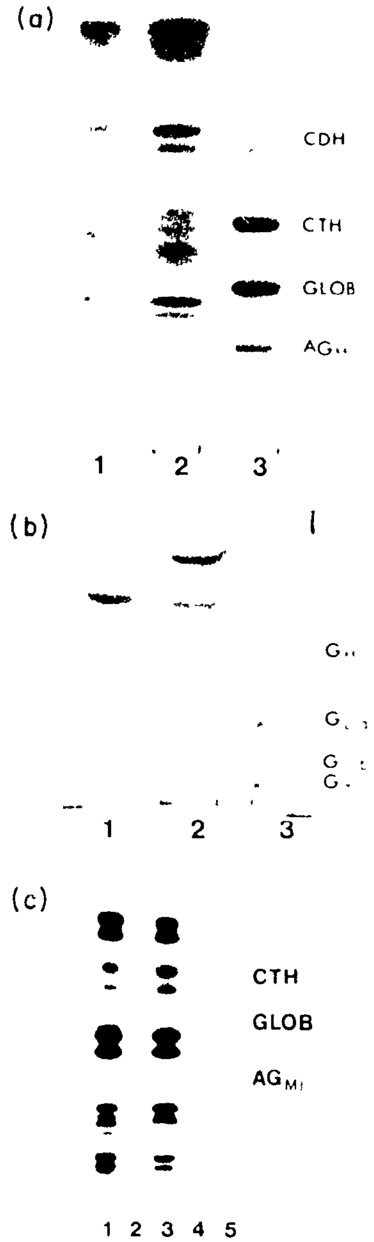

Figure 3 (a) Thin layer chromatogram of the neutral glycolipids from K562 cells Lane 1 K562 parent line lane 2, K562 Clone I lane 3 standards The standard markers are labelled Solvent system chloroform methanol-water (60 35 8) Detection with orcinol spray on HPTLC silica plates (Merck, Darmstadt, FRG) The nomenclature structure and mobilities of standard neutral glycolipids and gangliosides are as described by Suzuki *et al* (1981) The mobility of CMH (not shown) is above that of CDH (near the initial band of lanes 1 and 2) paragloboside, between that of globosides and AG_{M1} on the figure asialo G_{M2} between that of CTH and globosides (see Young *et al* 1981) (b) Thin layer chromatogram of the gangliosides from K562 cells Solvent system chloroform methanol water (55 45 10) containing 0.25% $CaCl_2$ Detection with resorcinol spray The G_{M2} and G_{M1} ganglioside standards (not shown) migrate above the position of G_{M1} on the figure (c) Autoradiogram of thin layer chromatogram of ^3H-labelled gangliosides Lanes 1 and 2, K562, lanes 3 and 4, Clone I, lanes 1 and 3, cells incubated with [^3H]galactose, lanes 2 and 4, cells incubated with [^3H]fucose, lane 5, standards

Table 1 Re[illegible] incorporation of [illegible] labelled [illegible] into cells

		Cell line		
Fraction measured	Tracer	K562	Clone I	[illegible]
Total cell protein*	Fucose	1.00	[illegible]	ND
Neutral glycolipid†	Ratio	[illegible]	[illegible]	[illegible]
	(Fuc:Gal) × 10‡	[illegible]	[illegible]	[illegible]
Gangliosides†	Ratio	[illegible]	[illegible]	[illegible]
	(Fuc:Gal) × 10‡	[illegible]	[illegible]	[illegible]

ND [illegible]

* [illegible] relative to parent K562

† [illegible]

‡ [illegible] parent K562

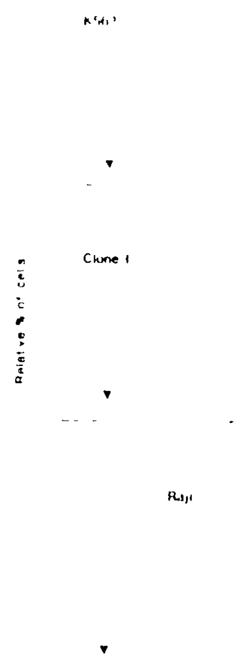

Figure 4 Analysis by flow cytometry of immunofluorescent staining of K562 and Clone I cells by antibody (H36 71) reactive with the lactoneofucopentaose III structure Horizontal axis is the fluorescence intensity, expres[illegible] that 10 of the smallest divisions represents a doubling of the intensity The vertical axis is the relative cell number normalized to percentage of total Raji is a B lymphoblastoid cell line lacking the H36 71 antigen and used here as a negative control cell

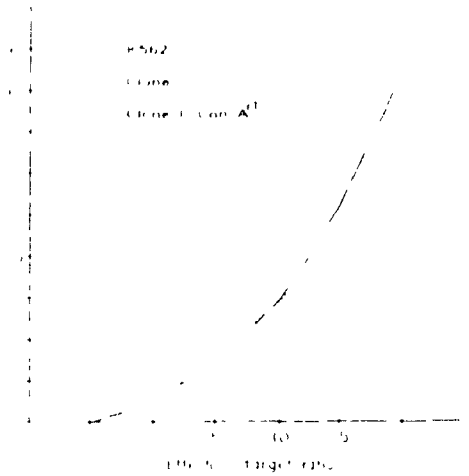

Figure 5. Representative profile of the sensitivity to NK lysis of sublines of Clone I made resistant to the toxic effects Con A. Sublines designated Clone I Con A^{r}

chromatographic patterns (not shown) were essentially identical between the Con A-resistant line and parent K562. The low mobility species seen in the products of Clone I were not visualized.

DISCUSSION

In this paper we have compared some of the plasma membrane properties of a human leukaemia cell line (K562) with those of a variant (K562 Clone I) selected for relative resistance to lysis by peripheral blood NK cells. None of the major externally oriented proteins accessible to radio-iodination by lactoperoxidase produced any differences in their band pattern on acrylamide gels including those peptides with sufficient mannose content to be enriched by affinity chromatography on immobilized Con A. Neither were any differences noted in the major sialylated glycoproteins labelled by the periodate–borohydride technique or the major fucosylated structures labelled by biosynthetic incorporation of tritiated fucose. These results indicate that the altered sensitivity of Clone I cells to NK was not the result of a gross change in the protein composition of the plasma membrane or in a general modification in the complexity of protein oligosaccharides. However, this does not negate the possibility that the changes relevant to NK may be subtle and might be visualized using more sensitive or specific probes.

Analysis of the glycolipids indicated that the overall pattern of these structures were very similar between the two K562 lines. However in both the neutral glycolipids and the gangliosides there was a minor group of bands of low mobility that were seen in the products of Clone I but not in those of parent K562. Incubation with radioactive fucose showed that Clone I incorporated several times more of the label into lipid than did K562. Since in both lines similar quantities of fucose were found in proteins of similar composition, it is unlikely that the lipid differences were due to variations in transport capability or pool size of the unlabelled monosaccharide. As shown in Fig. 3c, the predominant species of galactose-labelled gangliosides were identical in the two cells, but the minor bands that labelled with fucose were seen only in extracts of Clone I.

The Clone I variant was derived by depleting the parent population of conjugate forming cells and subsequently cloning the unbound fraction (MacDougall *et al.* 1983). Since the progeny of these cells were killed to the same extent as the parent by ADCC and competed less well against the original K562 in the cold target assay we proposed that they remained fully sensitive to cellular lytic mechanisms but bore either decreased blocked or otherwise ineffective target structures for the major population of unstimulated effector cells. That the defect was quite selective was suggested by the observation that interferon activated effectors killed Clone I as well as K562 (MacDougall *et al.* 1986). Were this resistance to NK due to a lack of one of the major Con A binding glycopeptides described by Roder *et al.* (1979) one would have expected that the proteins which eluted from columns of immobilized lectin would have been different between the two lines. Since this was not observed alternative explanations (for example failure of the relevant structure to bind to Con A Sepharose) or more sensitive techniques of analysis must be sought.

Recent work by Dennis and collaborators (Dennis & Laferte 1988) using a series of murine lines derived from the MDAY D2 lymphoma has shown that in contrast to the highly metastatic NK resistant parent cells a poorly-metastatic variant is highly sensitive to NK lysis. They have demonstrated further that the resistant cells bear a truncated form of a triantennary N-linked glycoprotein oligosaccharide which when isolated and added to a 4 hr cytotoxicity assay mediates a slight decrease in the lytic activity of stimulated cloned effector cells. Based on these findings they have proposed that the target structure for this type of NK cell is not a single homogeneous species but a broader group of Con A binding glycoconjugates. The data shown here with human leukaemic cells of myeloid derivation do not conform to this model, since there were not any striking differences between the sensitive and resistant K562 lines in the Con A binding of their major proteins.

On the other hand the Con A-resistant Clone I (Clone I Con A^{r}) returned to the original level of K562 sensitivity and lost the added group of fucosylated glycolipids. Likewise, Pohajdak has reported that a Con A-resistant Chinese hamster ovary cell line has increased sensitivity to murine NK cells (Pohajdak Wright & Greenberg 1984). This is consistent with the possibility that there may be glycolipids which can be modified to render a NK target structure inactive. A potential role for glycolipids in modulating the NK recognition process has been suggested by the work of Young *et al.* (1981) with another set of murine tumour lines. Although they observed a decrease in the quantity of asialo G_{M_2} in their NK-resistant line (cl 27av), direct lipid transfer did not reconstitute sensitivity. From this they concluded that the altered lipids were not the actual binding sites. Based on other studies in the mouse system, Yogeeswaran (1983) proposed that changes in cellular glycolipids including increased sialylation, may contribute to the neoplastic phenotype metastasis and the escape from immune surveillance. In the K562 model used here, the asialo G_{M_2} fraction was of similar intensity on the chromatograms of the two lines, and the decreased sensitivity of Clone I remained unchanged after treatment with neuraminidase (MacDougall *et al.*, 1986). Nonetheless, it is possible that modulation of the lipid environment surrounding a target protein may alter its conformation, exposure, or mobility within the membrane and lead to altered function. Such effects have been reported for receptors for

transferrin (Muller & Shinitzky 1979) and platelet derived growth factor (Bremer *et al* 1984). Although the positive findings in these K562 cells were observed in the glycolipids, it is possible that they simply are a reflection of other changes in a pathway used also for synthesis of peptide oligosaccharides. Indeed sensitivity of K562 to NK is protease sensitive but neither trypsin nor chymotrypsin decreases the killing of K562 or Clone I by protein A-activated effector cells (MacDougall *et al* 1986).

Although it is premature to conclude what the NK target structures may be, all of these observations continue to suggest that interactions with surface oligosaccharides may be essential components of the reaction or its regulation. These experiments on K562 cells demonstrate that variants arising from a tumour mass in the absence of added mutagen may require only minimal or indirect changes in their membrane composition to become resistant to NK mediated lysis.

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CONCLUSIONS

This series of publications presents the results of an integrated approach to NK effector-target recognition. An NK-resistant subline of the standard NK-susceptible target cell K562 has been derived and used to address the issue of target specificity, both biochemically and as it relates to NK cell activation.

Clone I, an NK-resistant variant of K562, was selected without prior exposure to mutagen by repeatedly depleting effector-target cell conjugates and cloning. Such cells can be killed at a level equal to that of the parent by complement and antibody-dependent cellular cytotoxic mechanisms indicating that they are resistant specifically to NK-mediated cytolysis and are not generally refractory to cytolytic mechanisms. Cold cell competition experiments show that Clone I is not able to compete as effectively as K562 for labelled K562 targets, although both K562 and Clone I compete equally well for labelled Clone I targets. Together, these data indicate that Clone I is specific in its resistance to a major population of unstimulated NK cells, probably through an absent or masked NK recognition site. Effector cells activated by staphylococcal protein A are able to kill K562 and Clone I equally well. Clone I and K562 share identical cell culture characteristics, morphology, karyotype profile, and a lack of expression of surface immunoglobulin, beta$_2$-microglobulin, HLA p44, or Ia-like antigens as determined by flourescence microscopy.

Together, these characteristics describe a cell line pair that may differ only in their susceptibility to NK cell recognition and cytolysis. The comparison of these cell

lines has led to a number of observations addressing several central issues in NK cell physiology.

Biochemistry of interactions between NK and target cells

A comparative analysis of the plasma membrane properties of K562 and Clone I cells has not revealed any differences in the expression of any of the major cell surface proteins, or in the nature of the protein oligosaccharides. This includes SDS-PAGE analysis of cell surface proteins accessible to lactoperoxidase-catalyzed radioiodination, proteins enriched by affinity chromatography on immobilized Concanavalin A, sialylated proteins labeled by the periodate-borohydride technique, or protein-bound oligosaccharides biosynthetically labelled by tritiated fucose. K562 and Clone I are identical in their levels of expression of lactoneofucopentaose III determinants (also known as SSEA-1 or CD15), and do not change their NK-sensitivity profile after incubation with neuraminidase. Further analysis of these cell lines has been undertaken by Bernier and Sullivan (16) using a variety of lectins as probes for different sugars. They have shown that nitrocellulose blots of proteins from plasma membrane enriched fractions of K562 and Clone I give identical staining patterns when either Con A (mannose), wheat germ agglutinin (WGA - N-acetylglucosamine/sialic acid), soybean agglutinin (SBA - N-acetylgalactosamine/galactose), peanut agglutinin (PNA - galactose), or Ulex europaeus agglutinin (UEA-1 - fucose) is used as a probe. Treatment of the membranes with neuraminidase before electrophoresis and nitrocellulose blotting did not unmask any

differences in the staining patterns. In their study, K562 and Clone I also were identical in their lectin resistance properties when assessed with the lectins Con A, WGA, UEA-1, and Ricinus cummunis lectin (RCA). Together, these data suggest that although Clone I appears to be resistant to a major population of NK cells, this resistance cannot be associated with any detectable change in cell surface glycoproteins.

Analysis of cellular glycolipids by thin layer chromatography indicated that in both the neutral and ganglioside fractions, the products of the NK-resistant Clone I variant contained a group of low mobility bands that were not seen in those of the parent K562. Clone I had a several-fold higher incorporation of tritiated fucose into both groups of glycolipids than did K562, and this incorporation was localized to those low mobility bands that were not present in the K562 parent. The Con A-resistant Clone I variant returned to the original level of K562 NK-sensitivity, and concurrently lost the added group of fucosylated, low mobility glycolipids. Thus, it is possible that these complex glycolipids are themselves NK target structures, and that the increased fucosylation could interfere with their recognition function and render the cells NK-resistant. However, the experiments described in **Publication 2** (Figs. 5,7 and 8) show that the sensitivity of K562 and Clone I to resting NK decreases markedly upon treatment of these target cells with the proteases trypsin or chymotrypsin. Although these data suggest that protein structures are involved in the recognition sequence, they do not preclude the possibility that the putative peptide NK target structure may require a distinct glycolipid environment in order for its function to be expressed. It is important to note that putative K562 target proteins isolated by Henkart et al (69) require reconstitution into endogenous

glycolipid micelles before they can inhibit effector-target interactions. Modulation of membrane glycolipids, such as those that occur in the Clone I cell, may render an NK target structure ineffective. Such a modulation of cell surface protein function by changes in the glycolipid environment has been reported for receptors for transferrin (125,134) and platelet-derived growth factor (23).

The NK-resistance of Clone I does not conform to any of the described models of NK target recognition, almost all of which focus on membrane proteins or glycoproteins. Both Roder et al (162,167), and Henkart et al (69) have described the isolation of Con A-binding glycopeptides that appear to inhibit effector-target interactions. Were the resistance of Clone I due to a lack of one of these glycopeptides, the proteins which eluted from the immobilized Con A columns should have been different between the two cell lines. No differences between K562 and Clone I Con A-binding proteins were observed.

Lactoneofucopentaose III structures (Le^x, SSEA-1 antigen or CD15) have been proposed as NK target structures. Harris et al (68) demonstrated that the ability of an anti-SSEA-1 monoclonal antibody to inhibit NK-mediated lysis of target cells was proportional to the expression of SSEA-1 on the cell surface. A more recent study has suggested that targets capable of binding NK cells express lactoneofucopentaose hapten, and do not express surface L-fucose residues capable of interacting with the Ulex europaeus agglutinin (223). Our data shows that K562 and the NK-resistant Clone I are equally reactive with a monoclonal antibody (H36/71) that recognizes the lactoneofucopentaose III structure. These cell lines incorporate tritiated fucose into protein equally well, and exhibit identical profiles when these proteins are separated by SDS-PAGE. Furthermore, Bernier and Sullivan (16) have shown that

plasma membrane fractions of Clone I and K562 are equally reactive with the Ulex europaeus lectin. Conversely, the Clone I Con A^{r1} cells are as sensitive to NK-mediated lysis as the original K562 parent, yet do not bear a detectable quantity of membrane SSEA-1 antigen. This clear dissociation between SSEA-1 antigen expression and NK sensitivity argues against this type of structure as a unique NK target molecule in K562 cells.

It has been suggested that the ganglioside G_{M2} is recognized as a target structure by natural killer cells. This is based on the ability of this ganglioside to specifically inhibit NK binding to K562 cells, as well as a correlation between the quantity of G_{M2} on a given target cell and its sensitivity to NK-mediated lysis (7). Thin layer chromatography of gangliosides did not reveal any differences in the intensity of the G_{M2} bands between K562 and Clone I.

Other cell lines have been described with altered sensitivity to NK-mediated cytolysis. The work of Dennis and Laferte with WGA-resistant mutants of the MDAY-D2 murine lymphoma (35,36), and the work of Ahrens and Ankel (3,4) with a series of lectin resistant mutants of Chinese hamster ovary (CHO) cells has demonstrated that in these systems natural killer cells recognize and kill targets depending on the nature of N-linked oligosaccharides present on the cell surface. Specifically, those mutants most sensitive to NK-mediated lysis (and subsequently less metastatic in the Dennis and Laferte model) expressed higher densities of high-mannose type, hybrid-type, or incomplete complex-type (overall decrease in galactose and/or sialic acid in glycoconjugates) oligosaccharides. Ahrens and Ankel (4) have subsequently shown that only high mannose-type glycopeptides and none of the complex-type block the lysis of NK targets. Many of these proposed NK target

structures are, or would be, capable of binding to Con A. The three K562 lines described here are similar in their complement of immobilized Con A-binding proteins, as well as in their expression of fucosylated and sialylated proteins as described in **Publication 3**. This suggests that the biochemical basis for the differences in their sensitivity to NK cells results from another mechanism.

The K562/Clone1/Clone I Con A^{r1} model can be instructive in that changes in susceptibility to NK-mediated cytolysis occur in parallel with changes in cellular glycolipids. While this model does not entirely negate any of those previously discussed, it raises the issue of how sensitivity to NK cells is determined. Our model suggests that target cell recognition is a more complex process than has been addressed in the literature. Clearly, a number of plasma membrane components acting in concert may determine whether a target is sensitive to NK cell-mediated lysis. Resistance to NK cell-mediated lysis may not have to be accompanied by a loss in some cell-surface glycoprotein component, even though this component may function as a requisite target structure. Furthermore, our model indicates how readily, and by what mechanisms a tumor cell may modulate its membrane components to avoid detection by natural killer cells.

Changes in specificity of natural killer cells after activation

It has been assumed that activation of NK cells with either IFN or Il-2 leads to an increase in the efficiency or vigor of the cytotoxic response (see **Regulation of NK**

cells) with little change in the recognition specificity of the response. The increase in cytotoxic efficiency has been used as an explanation for the increased range of target cell types following NK activation. This implies that susceptibility of a given target is not a recognition-limited event, but may be a result of differing thresholds of susceptibility to cytotoxic molecules. The issue of target cell specificity in relation to resting or augmented NK cells has not been resolved. The data presented and discussed in **Publication 2** suggest at least two possibilities. Although trypsin treatment of Clone I and K562 significantly reduced their sensitivity to basal NK, it did not affect sensitivity to augmented NK. Results in the single cell assay were similar; trypsin decreased conjugate formation and the fraction of bound cells that were killed by basal NK, but did not affect the increased lytic efficiency of augmented NK. Trypsinized targets, regardless of their equal sensitivity to lysis by augmented NK, were not as active competitors for augmented effectors. The most reasonable explanation for these data is that K562 cells exhibit surface structures that can trigger release of lytic molecules from augmented NK cells under conditions that are not sufficient to trigger their release from resting NK. Although this suggests that augmented NK may respond to a much smaller number of target molecules than resting NK, it is also possible that the two states of NK activity respond to different target structures. Clearly, the glycolipid structures present on Clone I that may be responsible for modulating the function of target structures for resting NK are irrelevant to augmented NK. A similar situation is presented by the trypsin treatment data (**Publication 2**, Fig. 6), where greater than 90% of the surface proteins accessible to lactoperoxidase catalyzed radioiodination were removed.

Analysis of target cell recognition by NK and LAK effectors has been attempted using K562 (NK target) and FEMX cells (LAK target) in cold-target competition assays (138). When K562 cells were used as the labelled target with fresh LGL, unlabelled K562 but not FEMX were able to inhibit the lysis of the labelled K562. The same pattern was obtained when LAK effectors were used. However, when FEMX cells were used as the labelled targets, FEMX and K562 competed equally well for both NK and LAK effectors. In further experiments, LAK effectors were separated into FEMX binders and nonbinders and then tested for lytic activity against K562 and FEMX. FEMX binding effectors lysed only FEMX, whereas both FEMX binding and nonbinding effectors lysed K562 targets. The data from these experiments were interpreted to indicate a difference in recognition structures involved in NK and LAK activity. However, the distinction between lymphokine-activated killers and augmented NK has not been clarified, and therefore differences in specificity between these two may also exist.

The characteristics of NKCF-mediated in contrast to perforin-mediated lysis suggests that a shift in the predominance of these lytic molecules may be a functional consequence of activation. The characteristics of NKCF-mediated killing appear to reflect those of resting NK cells: relatively slow kinetics of lysis and a limited range of specificities. The characteristics of perforin-mediated killing appear to more closely resemble those of augmented NK cells: rapid kinetics of lysis and a relative lack of specificity. Were target cell specificity demonstrated in both a recognition step and in the relative specificity of the lytic molecules themselves, then such a shift in the predominance of these lytic molecules would explain many of the observations previously discussed. The presence and activity of perforin has been

closely linked to cytolysis mediated by LAK and NK-derived cell lines.

The continued use of IL-2 dependent NK-clones and NK-derived cell lines without regard to their activated status, lack of a unified definition of NK cell activation, and a lack of knowledge about the molecules involved in effector-target interactions have impeded progress in the analysis of NK cell activation and target cell specificity. The use of closely related cell lines such as the K562/Clone I/Clone I-Con A^{rl} series described here offers an important alternative, and allows the complex issue of target cell specificity of augmented NK to be more effectively approached experimentally. In summary, the study of K562, the NK-resistant variant Clone I, and Clone I Con A^{rl} provides a unique model for the investigating NK effector-target interactions. Analysis of these variants, selected in the absence of mutagen, indicates that altered sensitivity to NK-mediated cytolysis may not necessarily be accompanied by major changes in the predominant cell-surface glycoprotein structures. The biochemical definition of NK-sensitivity may be complex and involve several plasma membrane components, including both proteins and glycolipids. Furthermore, the modulation of cellular glycolipids under selective environmental pressure may be one means whereby a tumor cell can escape surveillance by NK. Further investigation of these K562 variants should provide additional insight into the regulation of this mechanism as well as the mechanism of effector-target interactions of resting and augmented NK cells.

Statement of Originality

The three published papers contained herein represent the following contribution to original knowledge:

1. A subpopulation of cultured human leukemia cells contains spontaneous variants that are resistant to the cytotoxic effects of NK cells.

2. In this K562 model a quantitatively minor subgroup of fucoslyated glycolipids of Clone I and Clone I Con A^{rl} appears and disappears concurrently with changes in sensitivity to NK-mediated lysis. This association is suggestive of, but not proof that the glycolipids may be related to or modulate one or more of the early events of the recognition process.

3. The differential sensitivity between K562, trypsin-treated K562, and Clone I to unstimulated and augmented NK activities suggest that the early steps in the recognition mechanism are different for the two states of cytotoxic cell.

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