The University of Manitoba

BASIC FIBROBLAST GROWTH FACTOR-INDUCED PROLIFERATION IN MOUSE EMBRYONIC FIBROBLASTS AUGMENTS THEIR CYTOLYSIS BY NK CELL-DERIVED LYTIC GRANULES.

BY

SUSAN C. BATTISTUZZI

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LIST OF ABBREVIATIONS

NK.....natural killer
RNK.....rat natural killer
LGL.....large granular lymphocyte
PBL.....peripheral blood lymphocyte
TCR.....T cell receptor
MHC.....major histocompatibility
LAK.....lymphokine activated killer
IL.....interleukin
CTL.....cytotoxic T lymphocyte
IFN.....interferon
CSF.....colony stimulating factor
GM-CSF..granulocyte-macrophage colony stimulating factor
ADCC....antibody dependent cellular cytotoxicity
CAM.....cell adhesion molecule
LTBMC...long-term bone marrow culture
TGF.....transforming growth factor
TNF.....tumor necrosis factor
VSV.....vesicular somatitis virus
LCMV....lymphocytic choriomeningitis virus
MCMV....murine cytomegalovirus
NKCF....natural killer cytotoxic factor
BLT.....N,α-benzyloxycarbonyl-L-lysine thiobenzyl ester
RBC.....red blood cell
SRBC....sheep red blood cell
bFGF....basic fibroblast growth factor
EGF.....epidermal growth factor
DM.....defined medium
BSA.....bovine serum albumin
HBSS....Hank's balanced salt solution
PBS.....phosphate buffered saline
SE.....standard error

CHAPTER 1: ABSTRACT

NK cells preferentially lyse normal embryonic and undifferentiated target cells. Moreover, compared to mature differentiated cells, embryonic or immature cells are very responsive to growth factors and respond with a high rate of proliferation. Growth factor induced proliferation in fibroblasts was evaluated as a requirement for susceptibility to NK cell-mediated cytolysis. Basic-FGF-induced proliferation in 10T1/2 fibroblasts augmented their sensitivity to NK cell cytolysis. Basic-FGF did not enhance effector cell recognition of 10T1/2, but modified a post-binding lytic event.

NK cell granule-associated cytotoxins, thought to mediate post-binding cell lysis, were evaluated as the mediators of bFGF-enhanced cytotoxicity. We hypothesized that bFGF-induced proliferation in 10T1/2 fibroblasts modifies the ability of NK derived granule molecules to lyse these cells. 10T1/2 fibroblasts made quiescent in serum-free medium and 10T1/2 stimulated with bFGF were exposed to rat NK leukemia cell-derived granules. In ^{51}Cr cytotoxicity assays, granules failed to produce membrane damage in quiescent and bFGF-stimulated 10T1/2. However, in the cytotoxicity adhesion and ^{125}I-IUdR DNA fragmentation assays, quiescent 10T1/2 were moderately damaged by granules. Initiating proliferation with bFGF augmented

granule-induced loss of adhesion and DNA fragmentation. To determine whether granule-associated cytolysin was responsible for the cytolysis, 10T1/2 were exposed to heparin-agarose purified cytolysin and cytolysin-depleted granules. Purified cytolysin failed to produce significant ^{51}Cr release, loss of adhesion, or DNA fragmentation. Furthermore, selectively depleting cytolysin from granule preparations did not abolish loss of adherence or DNA fragmentation. Loss of adherence and DNA fragmentation, but not membrane damage, may represent mechanisms where bFGF-stimulated, proliferating 10T1/2 fibroblasts are preferentially lysed by NK cells. Basic-FGF modifies the ability of granule molecules, but not cytolysin, to lyse 10T1/2 fibroblasts.

CHAPTER 2: REVIEW OF RELEVANT LITERATURE

A. NATURAL KILLER CELLS

1. Introduction

The observation that lymphocytes from normal, un-immunized individuals could lyse tumor cells initiated the study of the natural killer (NK) cell. NK cells are lymphocytes that participate in natural, cell-mediated cytotoxicity, which, unlike T lymphocyte-mediated cytolysis, proceeds without prior antigenic sensitization (78,107). NK cells are large granular lymphocytes (LGL) that comprise 5% of human peripheral blood lymphocytes (PBL). LGLs with NK cytotoxicity express CD16, NKH1, CD2 antigens in humans, and express LGL-1, NK1.1, asialo-GM1 in mice. NK cells lack the capacity to rearrange and express either alpha/beta, or gamma/delta T cell receptor (TCR) genes, and lack the TCR-associated CD3 surface antigen. Natural cytotoxicity occurs without major histo-compatibility complex (MHC) restrictions, and NK cells do not recognize their targets through the TCR/CD3 complex. NK cells are produced by, and mature in an intact bone marrow environment. The cells, widely distributed in the body, occur in the spleen, peripheral blood, lungs, liver, and gut (54,78,88,107,125,153,166,169).

A small population of lymphocytes resembles the NK cell, because the cells have LGL morphology, express NK-

associated surface antigens, and mediate spontaneous, MHC-unrestricted cytotoxicity. In contrast to "true" NK cells, these effector lymphocytes are TCR^+ and $CD3^+$, and recognize target cells through the TCR/CD3 complex. Some investigators differentiate the two populations: NK cells are TCR^-, $CD3^-$ cells, and the TCR^+, $CD3^+$ cells are T lymphocytes that express "NK-like" cytotoxicity (78,88). An additional mediator of natural, MHC-unrestricted cytotoxicity is the lymphokine activated killer (LAK) cell. LAK cells are PBLs, bone marrow-derived cells, or splenocytes in vitro stimulated with interleukin-2 (IL-2). PBLs, cultured with IL-2 for at least 10-12 days, develop LAK activity and express a $CD3^+$, $CD16^-$, $CD56^-$ phenotype (131). Only in long term cultures do T lymphocytes contribute significantly to LAK activity. Whereas in short term cultures, from 2 to 5 days, LAK cells are predominantly NK cells. These cells and their precursors express LGL morphology, NK cell-associated surface antigens but do not express T cell-associated antigens such as the TCR (60,78,88,141).

2. Morphology and Cytochemistry

In humans, rodents and all vertebrates tested, at least 90% of NK cell activity is associated with a small and fairly discrete population of lymphocytes, the large

granular lymphocyte. In the human system, the LGL represents about 5% of PBLs, and 1-3% of mononuclear cells (107). Although a small proportion is associated with the cytotoxic T lymphocyte (CTL), LGLS do not appear to represent immature T cells (88). However, one group of investigators has found that thymocytes give rise to NK cells (102). However, it remains unclear whether these NK precursors are truly T cell precursors, or independent NK cell precursors that occur in the thymus. A percentage of LGLs do not express NK cell activity and it remains unclear whether these cells are capable of natural cytotoxicity (13,107).

LGLs exhibit morphological and cytochemical features that differentiate them from other lymphocytes, from macrophages, and from granulocytes. LGLs, 16-20 μm in diameter, have an extended cytoplasm, giving the cells a high cytoplasm-to-nuclear ratio (107,129,153). LGLs possess an irregularly shaped, lobed, or indented nucleus (153,161). LGLs are not phagocytes and unless stimulated with IL-2, interferons (IFN), or bacterial products, they do not adhere to nylon wool and plastic surfaces (107).

The outstanding morphological feature of LGLs is azurophilic staining granules present in their cytoplasm (153,161). Identified by electron microscopy, the granules appear as electron dense structures, enclosed by a unit

membrane (59,99,166). The granules occur in close physical association with the Golgi complex (129,161). Participants in the NK cytolytic process, azurophilic granules are primary lysosomes (59,99,166). They contain lysosomal enzymes: beta-glucuronidase, acid phosphatase, aryl-sulfatase and trimetaphosphatase (24,59,99,153,161). Like lysosomes, granule activity is sensitive to the effects of lysosomotropic reagents such as chloroquine (166). Unlike the granules of macrophages and granulocytes, LGL granules do not contain non-specific proteases, peroxidase, lysozyme, histamine, and heparin (99,107,129,161). LGL azurophilic granules express and are identified by serine esterases, chondroitan sulfate proteoglycans, and the hemolytic protein cytolysin/perforin (166,169).

F344 rats are susceptible to a form of leukemia, where the leukemic cells express both LGL morphology and NK cell cytotoxicity. The leukemic rat NK (RNK) cells closely resemble normal F344 rat peripheral blood LGLs in structure and cytochemistry (99,161). The RNK leukemia is easily transplanted into F344 rats and grows rapidly in the host, in the spleen or as ascites. Isolated RNK cells show high natural cytotoxicity and in some cases, antibody-dependent cellular cytotoxicity (ADCC). The granules are isolated from RNK cells by density gradient centrifugation; the isolated granule material expresses the same lytic

potential of the intact cells. The RNK leukemia cell line remains an excellent model for studying NK cells and NK cytotoxicity (59,99,161).

3. Surface Antigens

Lymphoid- and myeloid-derived cells carry antigenic molecules on their external plasma membranes. While lymphoid and myeloid cells, including NK cells, carry unique surface markers, each population shares some markers with each other. According to Lanier et al, there is no reason why a particular surface antigen has to be expressed on exclusively one cell lineage (78). Any combination of surface antigens, unique and common, helps to identify a particular cell population from others (78,107). Human and experimental animal NK cells express and can be identified by their own combination of surface antigens.

3.1. Surface Antigens of Human NK Cells

NKH1, also known as Leu-19 or CD56, is a 200 kD surface antigen found on resting and activated human NK cells (125). A population of T lymphocytes, including some clones and long-term cultured T lymphocytes, express the antigen. The T cells that present NKH1 belong to the $CD3^+$, TCR^+ cells expressing NK-like cytotoxicity. NKH1 nevertheless, occurs almost exclusively on NK cells

and is found on more than 95% of NK cells (125,153). NKH1 may participate in NK cell-target cell binding (113). Approximately 50-70% of human NK cells express the surface antigen HNK-1, also called Leu-7 or CD57 (125,153). The 110 kD molecule is not, like NKH1, exclusively presented on NK cells. It appears on other lymphoid, myeloid, and non-hematopoietic cells such as adenocarcinoma and neuro-endocrine cells. HNK-1 occurs on some $CD3^+$, $CD8^+$ T lymphocytes but rarely on $CD4^+$ T lymphocytes. The function of HNK-1 is not known and the antigen's expression rapidly disappears from NK cells upon <u>in vitro</u> activation.

Human NK cells express the Fcγ receptor type III (7,153). The receptor, the CD16 antigen, is a 50-70 kD molecule expressed on 80-90% of human NK cells (125). Recent observations have demonstrated that CD16 is associated with the zeta chain, the only portion of the TCR complex found to be expressed on human NK cells (7,79). While T lymphocytes do not present CD16, granulocytes do. Macrophages and B lymphocytes carry an Fcγ receptor, but since it does not cross-react with anti-CD16 antibodies, it is structurally different from CD16. Most lymphocytes capable of spontaneous cytotoxicity express CD16. Since anti-CD16 antibodies inhibit ADCC, the antigen may be directly involved in the cytolytic reaction (125,153).

Human NK cells express the Leu-CAM (cell adhesion molecules) surface antigen family, also known as the β_2 integrins (43,113,125,153). On all cells which express these antigens, the β_2 integrins occur as a non-covalent heterodimer made of β_2 (CD18) and α subunits. The β_2 integrins include CD11a/CD18 (CD11a also known as LFA-1), CD11b/CD18 and CD11c/CD18. Although T lymphocytes express predominantly CD11a/CD18, granulocytes CD11b/CD18, and macrophages CD11c/CD18, the three β_2 integrin molecules are present on 80-95% of NK cells (113,125,153). Current observations indicate that the β_2 integrins participate in the NK cell's binding to and cytolysis of target cells (113,125).

CD2, a T lymphocyte-associated surface antigen, is expressed on 80-90% of human NK cells. CD2 is a 50 kD molecule composed of several epitopes including $T11_1$, $T11_2$, $T11_3$. All three epitopes occur on NK cells. CD2, $T11_1$ in particular, participates in forming rosettes with sheep erythrocytes (113,125). In conjunction with β_2 integrins, CD2 participates in adhesion of NK and target cell, and in activation of the NK cell for cytolysis (113,125,165).

3.2. Surface Antigens of Experimental Animal NK Cells

Like NK cells from the human, NK cells from animals carry surface antigens that help to distinguish them from

other lymphoid and myeloid cells (78,153). Murine NK cells express the surface antigens NK1.1 and NK2.1 (153). NK1.1 and NK2.1 are alloantigens and are not found in all mice but only expressed on NK cells from certain mouse strains. These surface markers have no clear function; however, NK1.1 expression is associated with high NK cytotoxic activity (78). Murine NK cells express an Fcγ receptor, much like CD16 on human NK cells (153). Murine Fcγ receptor similarly participates in ADCC reactions. Murine NK cells express an 87 kD surface molecule, LGL-1 (93). Unlike NK1.1 and 2.1, LGL-1 is non-allelic and occurs on the NK cells of all tested strains. Because LGL-1$^-$ NK cells possess little or no cytotoxic activity, the surface molecule may be related to NK-mediated cytotoxicity. The surface marker asialo-GM1 occurs on murine NK cells (107,153). Asialo-GM1, like LGL-1, is non-allelic and is expressed by multiple strains of mice. This antigen does not exclusively occur on NK cells. Asialo-GM1 is commonly found on other lymphocytes such as CTLs and on activated macrophages (93,153). Approximately 50-70% of murine NK cells express the antigen Thy-1 and 20% express Lyt-1. T lymphocyte specific surface markers expressed by murine NK cells include: Qa-2, Qa-4, Qa-5, Lyt-6, Lyt-10 and CR3 (93,120,153).

3.3. CD3 and the T Cell Receptor

T lymphocytes express the CD3-TCR complex on their cell surface (74,142). CD3 is a molecular complex composed of four protein chains: gamma, delta, epsilon, and zeta chains, and expressed as $\gamma\delta\epsilon\epsilon\zeta\zeta$. The TCR is expressed as a heterodimer and may include either alpha and beta or delta and gamma protein chains. T cells express either the alpha/beta TCR or the gamma/delta TCR, but not both (142). CD3 co-precipitates with the TCR proteins and TCR^- mutant T cells lack CD3 (74). Such observations indicate that while CD3 and TCR are non-covalently associated, expression of CD3 appears to be linked with expression of the TCR. During T lymphocyte ontogeny, the TCR genes are sequentially rearranged and expressed in conjunction with the CD3 genes (114). Unlike T lymphocytes, human, mouse and rat NK cells do not express the entire CD3-TCR complex (78,107,153).

Anti-CD3 or anti-TCR antibodies inhibit CTL-mediated target cell recognition, binding and cytolysis indicating that the CD3-TCR complex participates in CTL-mediated cytotoxicity. On the other hand, because anti-CD3 or anti-TCR antibodies do not inhibit NK-mediated cytotoxicity and because the total molecular complex is not expressed on human and animal NK cells, the NK cytolytic process involves surface antigens other than CD3-TCR (107).

Several investigators have observed that the CD3 epsilon chain gene is present in fresh and cultured NK cells but the gene is not expressed (78). Moreover, there is no evidence that genes of the CD3 gamma and delta molecules exist in NK cells. However, recent experiments have demonstrated that human $CD3^-$, TCR^- NK cells express the zeta protein and this molecule is presented as a complex with the CD16 antigen ($Fc\gamma$ receptor III) (7,8,79). It has been suggested that zeta antigen, in concert with CD16, is responsible for the activation of ADCC functions, and may regulate signal transduction in the activation of NK cells (7,79). While NK cells express CD3 zeta protein, these cells do not express the entire CD3 molecular complex and virtually all natural cytotoxicity appears to be mediated by $CD3^-$ lymphocytes (78,88,153).

No experiments have revealed the sequential rearrangement and expression of the TCR genes in human and animal NK cells (78). Current studies fail to demonstrate, in fresh and cultured NK cells, rearrangement of either alpha, beta, or gamma TCR genes (125,153). NK cells don't express TCR alpha or gamma mRNA, but some NK cells express mRNA for the beta molecule. However, NK-associated beta mRNA is a truncated and non-functional species (78,125). Finally, NK cells lack TCR proteins: neither alpha nor beta TCR proteins have been found in, or on NK cells, and

there is no evidence for the presence of gamma or delta TCR proteins (125).

4. Bone Marrow Origin of NK Cells

While B lymphocytes and myeloid-derived cells develop in the bone marrow and T lymphocytes develop in the thymus, current studies indicate that the NK cell originates and at least partially, matures in the bone marrow (54,78,90,129,142,153,158). In vitro experiments show that mature, cytotoxic NK cells originate from bone marrow cultures (153). Bone marrow cultures depleted of mature NK activity generate NK cytotoxicity with IL-2 stimulation (90,153). The generated effector cells have LGL morphology, express NK-associated surface markers NKH1 and CD16, lack T and B cell-associated antigens CD3, CD4, CD8, surface immunoglobulin, and CD19. That is, the cells are mature NK cells (90). Long-term rat bone marrow cultures generate pure, mature NK cells that express LGL morphology, NK-associated surface antigens and cytotoxicity against NK-sensitive target cells (158). In vitro cultured thymus cells, by contrast, produce mainly $CD3^+$ CTLs (153). Kumar has demonstrated that the thymus contains mature NK cells, but not NK cell precursors (reviewed in 88). The immature thymocytes develop into mature T lymphocytes, not NK cells.

In vivo studies additionally demonstrate that bone marrow appears to be the site of NK cell production. The thymus, as the source of NK cells, is ruled out because athymic, nude mice are deficient of T lymphocytes but possess NK cells. Athymic, nude mice express greater NK activity than their euthymic littermates (54,107,153,158). Microphthalmic (mi/mi) mice have a congenital osteopetrotic defect and express a defective bone marrow (73,153). The mice express a deficiency in NK cell maturation, and natural cytotoxicity. Experimentally reducing the bone marrow with ^{89}Sr, a bone seeking radioisotope, specifically decreases mature NK cell activity, without altering CTL and macrophage functions (120,153,158). Lethally irradiated mice are bone marrow- and NK cell-deficient but NK cell activity is restored with marrow transplants (120,153). The recipient's reconstituted level of NK cytotoxicity parallels the level of NK cytotoxicity of the donated bone marrow, demonstrating that the NK cell activity is intrinsically determined by the bone marrow's phenotype, not the genetic background of the recipient. The in vitro and in vivo data indicate that the differentiation of mature NK cells depends entirely on an intact and functional bone marrow environment (78,90,120,158).

5. Tissue Distribution of NK Cells

In humans and experimental animals, the NK cells have a wide tissue distribution. The spleen, while not a significant NK cell producer, contains a considerable quantity of the cells. The spleen relies almost entirely on cells emigrating from the bone marrow (107,120,153). Like the spleen, peripheral blood carries NK cells. NK cells are associated with mucosal epithelial tissues, particularly the gut epithelium, and populate the air-spaces and interstitial compartments of the lungs. Lung-associated NK cells often are responsive to locally derived regulatory factors. NK cells are present in liver. Liver-associated NK cells generally reside in non-parenchymal tissues. The lymph nodes and tonsils of healthy, normal individuals generally do not contain NK cells (107,153).

6. Lineage

Some investigators have suggested that NK cells represent a "function" mediated by several effector cells, including LGLs, T lymphocytes, macrophages, and granulocytes. Recent observations indicate that NK cells actually are a distinct lymphocyte population that expresses physiological and functional heterogeneity (107). While this does not adequately define a cell lineage, NK cells' morphological, physical, and functional properties

indicate they are not simply a function, but are a distinct cell population (78,107). Whether the NK cells belong to a unique and independent lineage, nevertheless, remains unanswered. While the NK cell appears to be a distinct population of cells, it shares enough properties with other lymphoid and myeloid cells to make one believe it derives from any of these lineages. Ortaldo describes several possible origins for the NK cell: (1) NK cells derive from the monocyte/granulocyte lineage; (2) NK cells derive from the T lymphocyte lineage; (3) NK cells, because they do not neatly fit into the above categories, derive from an independent, but still unidentified lineage (107).

While macrophages and granulocytes also arise from bone marrow precursors, morphological and cytochemical evidence contradicts the possibility that NK cells develop from a precursor of the monocyte/granulocyte lineage. NK cells share few characteristics with macrophages and granulocytes. NK cells, unlike macrophages and granulocytes, are non-phagocytic and unless stimulated with IL-2 or IFN, are non-adherent (107,129). NK cell lytic granules lack macrophage or granulocyte granule constituents and NK granules contain molecules not generally found in these other cells (99,129). Rat LGLs lack receptors for and are unresponsive to granulocyte-macrophage colony stimulating factors (107). W/W^{v} mice express a severe and intrinsic

defect in multipotent myeloid stem cells. The bone marrow of these mice cannot produce monocytes and granulocytes, but carry NK progenitors. The bone marrow from W/W^V mice is capable of developing and expressing normal, mature NK cell activity (78). While the defective bone marrow cannot produce myelomonocytic cells, it carries normal NK cell progenitors that develop into the mature effector cell, indicating NK cells can mature independently of myeloid stem cells, and may not belong to the myeloid lineage.

The second possibility suggests that NK cells derive from the T lymphocyte lineage. Some T lymphocytes express natural, MHC-unrestricted cytotoxicity and possess LGL morphology. CTLs and NK cells have similar lytic mechanisms, including granule cytochemicals, activation pathways, adhesion molecules, and methods of target cell damage (13,153,166,169). However, certain qualities indicate T cells and NK cells are unrelated regarding their lineage. The thymus produces T lymphocytes; the bone marrow produces NK cells (78,90,153). Mice treated with ^{89}Sr lose their bone marrow and NK cells but express normal T lymphocyte activity (13,158). Additionally, athymic, nude mice, despite lacking mature T lymphocytes, express high NK cell activity (90,120,153,158). On the other hand, Mingari et al have found $CD3^-$, $CD4^-$ thymocytes which under appropriate culture conditions including IL-2

stimulation, develop into $CD3^-$, $CD16^+$, $CD56^+$ cells resembling NK cells (102). However, it remains unclear whether the NK cells develop from precursors that also give rise to mature T lymphocytes. That is, there may exist in the thymus, precursors which give rise specifically to NK cells and remain independent of precursors that develop into T cells. Thus NK cells still belong to a separate lineage. Furthermore, the previous observations from bone marrow- and thymus-deficient mice cannot be overlooked. Additionally, Mathieson transferred liver-derived NK cells into the thymus of Ly5 mice, and found that the liver NK cells did not develop into thymocytes of donor phenotype (reviewed in 88). This suggests that NK cells may not represent immature T lymphocytes. Severe combined immune deficient, or scid mice express a defect in the early differentiation of B and T lymphocytes, and lack mature B and T lymphocytes (78). The bone marrow of scid mice contained normal quantities of NK cell progenitors. These progenitors were capable of developing into mature NK cells, despite the inability of the B and T lymphocyte precursors to mature. Finally, Kumar has shown that scid mouse-derived thymus cells, upon IL-2 stimulation, develop into T lymphocytes but do not develop into NK cells (88). He suggested that, while the thymus may contain mature NK cells and T lymphocyte precursors, it does not contain NK

cell progenitors, and this directly contradicts the data presented by Mingari's group (102). Mature NK cells can develop independently of other lymphocytes, including T lymphocytes. Normal NK precursor cells can be dissociated from the T cell precursors and no convincing evidence exists to suggest NK cells derive from and belong to the T lymphocyte lineage (107). While the hypothesis that NK cells constitute a third, independent lineage has not been established, these cells cannot easily fit into either myeloid or T lymphocyte lineages (78,107).

An NK-specific stem cell has not been isolated and the identification of discrete maturation stages, which describe the cell lineage from a stem cell to mature cell, have not been demonstrated (78,120). However, Pollack et al have identified transplantable murine bone marrow derived progenitor cells that restore NK cell activity in NK-deficient mice (120). One progentitor is a large and proliferating cell which gives rise to a second progenitor, a post-mitotic cell. The post-mitotic cell may represent the most mature bone marrow species; upon discharge from the marrow, it migrates to the spleen. Both progenitors are related to mature murine NK cells since they express NK1.1 antigen and bind NK-sensitive YAC-1 cells but not NK-resistant P815 cells. Van den Brink et al have demonstrated that long term rat bone marrow cultures

(LTBMC) contain precursor cells that develop into populations of pure, mature NK cells (158). The precursor cells lack asialo-GM1 antigen, but express this rodent NK cell surface markers upon maturation. This group has not observed LTBMC cells to give rise to either mature B lymphocytes or myelomonocytic cells.

7. Regulation of NK Cell Activity

NK cells are positively and negatively regulated by cytokines, certain chemicals and cells. NK cells are positively regulated in vitro and in vivo by the interferon family, including IFNα, IFNβ, and IFNγ (24,107,153). IFNs increase the number of NK cells with lytic capability and increase the proportion of NK cells that bind to target cells, including NK-insensitive targets. Among NK cells that can conjugate with target cells, IFNs enhance the kinetics of cytolysis and induce NK cells to recycle after one round of lysis (107,153). Mice, stimulated in vivo with IFN, carry NK cells expressing blast formation and DNA synthesis (153). Polyinosinic: polyciticylic (poly I:C), some viral infections (including lymphocytic choriomeningitis virus) and the bacteria Coryneform parvum activate NK cells by stimulating the intrinsic production of IFN in the NK cells (107,153,163).

Like IFNs, IL-2 positively regulates NK cell activity

in vitro and in vivo (107). The IL-2-induced cellular responses include: proliferation of NK cells, increases in cytotoxicity against NK-sensitive and NK-resistant target cells and lymphokine activated killer activity (141,153). Normally non-adherent, NK cells upon IL-2 stimulation adhere to plastic and nylon wool. IL-2 stimulated NK cells develop increased locomotor activity, and secrete IFNs. IL-2 and IFN synergize in NK cell activation.

Several chemical mediators can activate or inhibit NK cell activity in vitro and in vivo. Bacterial products activate NK cells but mitogens, such as concanavalin A and phytohemagglutinin have no effect (78,107). Phorbol esters, prostaglandins (PG_E), cyclic nucleotides (cAMP), cyclophosphamide, and corticosteroids inhibit NK cell activity (107,153). Factors that inhibit NK cell activity at the target cell binding stage include EDTA, phorbol esters, and proteases. Other factors inhibit NK cytotoxicity at the post-binding lytic stage and may affect NK cell activation, kinetics of lysis, secretion of cytotoxins, and recycling. Cholera toxin, cAMP, PG_E, and some phosphate sugars inhibit post-binding events of NK cell activity (88,107). Transforming growth factor-beta (TGFβ), granulocyte-macrophage colony stimulating factor (GM-CSF), and IL-3 appear to block NK cell maturation in the bone marrow (153).

NK cells are also negatively regulated by cell-to-cell interactions (107). Both adherent and non-adherent suppressor cells affect NK activity; for example, rodent NK cells are inhibited by suppressor macrophages and lymphocytes. Additionally, Ofir et al have found that leukemic cells from mice infected with the Moloney murine leukemia virus directly suppressed NK cell activity. Even NK cells activated by the murine cytomegalovirus were down-regulated by the leukemic tumor cells (106). This data indicate that both immune cells and cells not associated with host immune responses appear to regulate NK cell function.

8. Function of NK Cells

NK cells perform cytotoxic and non-cytotoxic functions. The NK cell is a prime mediator of natural cell-mediated cytolytic reactions but also participates either directly or indirectly in immunoregulation. The exact in vivo role of these effector cells has not been established but their function has been demonstrated through in vitro and indirect in vivo observations (125). NK cell functions most likely are mediated by members of one discrete population of cells, rather than by cells from different lineages (107,125).

As mediators of cellular cytotoxicity, NK cells lyse a

broad family of target cells, including: undifferentiated, immature cells; tumorigenic and transformed cells; cells infected with virus or bacteria; particular fungi and parasites (88,107,153,163). The properties that make a particular cell susceptible to NK-mediated cytolysis are unresolved but for lysis to occur the NK cell must be capable of recognizing and adhering to the target (153). Additionally, the target cell must be able to activate the NK cell's lytic apparatus. The time required for NK cells to effectively lyse particular target cells varies. NK cells lyse YAC-1 lymphoma cells within one hour, but require up to 24 hours to lyse fibroblast targets (13,59).

8.1. Cytotoxicity Against Undifferentiated Cells

Some investigators have designated NK cells as mediators in the surveillance of primitive cells because the effector cells preferentially lyse immature rather than mature cells (107,153). Mature lymphoid cells are resistant to NK-mediated cytolysis but NK cells readily lyse immature thymocytes, undifferentiated bone marrow cells, and hematopoietic stem cells. In contrast to fully differentiated carcinoma cells, embryonic carcinomas are susceptible to NK cytotoxicity. Experimentally inducing differentiation in NK-sensitive K562 and U937 cells increases their resistance against cytolysis (153).

However, the relationship between NK-sensitivity and target cell maturity is not absolute. Phorbol esters induce differentiation in certain target cells such as K562 and with this treatment the cells' NK susceptibility is enhanced rather than depressed. Additionally, NK cells lyse some normal, differentiated fibroblasts. While certain target cells are more NK-senstitive with decreasing maturity, the level of cellular maturity remains an insufficient determinant of NK-sensitivity.

8.2. Cytotoxicity Against Tumor Cells

Athymic, nude mice lack T lymphocytes but express high NK cell activity. Investigators have found that compared to normal mice, nude mice do not express a greater incidence of primary spontaneous neoplasia, some virally-induced neoplasia or chemically-induced neoplasia (16,134). Certain transplanted tumor cells do not readily metastasize in athymic, nude mice (150). On the other hand, NK cell-deficient *beige* mice experience augmented tumor growth and metastasis (16). These observations imply that NK cells may mediate some defence against neoplastic and metastatic cells (13,153). Warner and Dennert proposed that the NK cell has the following functions of *in vivo* immune surveillance: inhibition of primary tumorigenesis, and resistance against tumor metastasis (16).

With *in vitro* assays, NK cells are capable of lysing certain tumor cells such as the YAC-1 lymphoma (166). Lymphokine activated killer (LAK) cells also lyse traditionally NK-resistant tumor cells such as fresh autologous solid tumors, leukemic blasts and allogeneic and xenogeneic tumor cells (141,153). Schwarz et al isolated LGLs which upon IL-2 stimulation adhered to plastic. They further expanded these cells with IL-2, thus generating LAK cells from a relatively pure population of NK cells (141). Compared with the conventional LAK cells, these effector cells expressed much greater *in vitro* tumor cell cytotoxicity.

Several investigators have observed that target cells transformed by oncogenes or viruses experience greater NK-mediated cytolysis than their untransformed counterparts. Normal fibroblast cell lines transformed with either Ha-*ras* or Ki-*ras* oncogenes develop a tumorigenic profile: they express anchorage-independent growth; they grow as tumors in mice; they exhibit spontaneous metastasis in mice (40,50). Some fibroblast cell lines such as 10T1/2 and Rat-1, when transformed by Ha-*ras* or Ki-*ras* oncogenes, experience increased *in vitro* susceptibility to NK-mediated cytolysis (50,51,64,65,152). Additionally, certain strains of adenovirus neoplastically transform rodent and hamster cell lines *in vitro* with the expression of the

viral E1A genes (32). Expression of the E1A gene, like expression of ras, augments in vitro NK-mediated cytolysis of the transformed cells.

However, ras-transformed NIH 3T3 fibroblasts do not experience greater susceptibility than the untransformed 3T3 cells to cytolysis by NK cells (51). Furthermore, E1A virus gene expression can increase the target cells' NK-sensitivity without transformation of the target (32). These observations indicate that the augmented susceptibility to NK cells occurs independent of cellular transformation and may be the result of other unknown cellular properties.

That NK cells have an in vivo anti-tumor role was suggested when tumor cells expressed increased growth and metastasis in NK-deficient beige mice but not in T cell-deficient nude mice (16,150). Talmage et al found that in contrast to normal mice, transplanted NK-sensitive B16 melanoma cells grew and spontaneously metastasized more rapidly in the NK cell-deficient beige mouse (150). NK-resistant B16 melanoma cells grew and metastasized at the same rate in both normal and beige mice. Ha-ras transformed 10T1/2 fibroblasts expressing a tumorigenic phenotype exhibited greater metastatic potential in beige mice than in their NK replete, normal littermates (40,50,51). Irradiated mice subsequently transplanted

with bone marrow express the NK cell activity of the donated marrow (54). Mice receiving bone marrow with high NK cell activity resisted and eliminated transplanted YAC-1 lymphoma cells. On the other hand, mice receiving bone marrow with low NK activity were more susceptible to the YAC-1 lymphoma.

A monoclonal antibody to the surface antigen NK1.1 depletes virtually all NK cell activity in mice but leaves other lymphocytes and immune functions intact (143). The lungs of mice treated this way experienced increased localization and growth with transplanted NK-sensitive B16 melanoma cells. Other NK cell destructive treatments, such as cyclophosphamide or anti-asialo-GM1 antibodies, produced similar effects on in vivo tumor survival. Standard LAK cells and Schwarz's LAK cells (expanded from pure NK cells) expressed in vivo anti-metastatic activity against tumor cells in experimental animals (141,153). Additionally, the LAK cells exhibited prolonged surveillance demonstrated by the increased life spans of the tumor-bearing experimental animals.

While NK cells appear to participate in the in vivo tumor defence, the anti-tumor activity of NK cells in human cancer patients is less clear. Immune-deficient individuals have approximately a 200 fold greater frequency of cancers than expected in the normal population (126).

Individuals with the X-linked recessive, combined variable immune-deficiency disorder (XLP), when infected with the Epstein-Barr virus, frequently develop malignant lymphomas. Patients severely immune-deficient as a result of HIV infection are highly susceptible to Kaposi's sarcoma, a cancer which is rare in the normal population. However, the immune-suppression in both XLP disorder and HIV infections involves T lymphocyte functions and the role of NK cells in the onset of these cancers is obscure. Furthermore, NK cell deficient Chediak-Higashi Syndrome patients, unlike beige mice, do not express a greater incidence of developing cancer (153). On the other hand, in familial melanoma, a disease with a hereditary predisposition, relatives at high risk of developing the disease express decreased NK cell activity. Patients who have developed primary, non-invasive melanoma similarly exhibit low NK cell activity. But it remains unclear whether the low NK cell levels have any bearing on the development of melanoma in these individuals. These studies do not directly link NK cell activity to the onset of, or resistance to certain human cancers. Neoplasia itself may suppress immune functions and the radiotherapy and chemotherapeutic reagents used in cancer treatment also deplete host immune functions (126).

8.3. Cytotoxicity Against Virus-Infected Cells

While T lymphocyte- and antibody-mediated immune responses are classically the major defense against virus infections, NK cells may also participate. Virus-infected cells, compared to uninfected cells, demonstrate greater susceptibility to *in vitro*, and possibly to *in vivo* NK-mediated cytolysis (153,163). Rodent and hamster embryo cells infected with certain strains of human adenovirus are more susceptible than uninfected target cells to lysis by syngeneic NK cells (32). Paya has demonstrated that $CD16^+$, $CD3^-$ LGLs produced tumor necrosis factor-alpha (TNFα) in response to vesicular somatitis virus (VSV)-infected target cells; antibodies against TNFα abolished the effect (reviewed in 88). Human influenza virus neuraminidases augmented *in vitro* NK-mediated cytotoxicity against K562 target cells (9). The stimulation is produced by the viral antigens and is independent of virus-induced IFN production. Other viral glycoproteins, for example, hemagglutinin from mumps, measles, and Sendai viruses, augment NK cell activity (163). This NK cell stimulation is inhibited by the addition of antibodies against neuraminidase or hemagglutinin (9,163). Welsh has found that NK cells respond chemotactically to virus-infected tissue exudates and to virally-produced IFNα and IFNβ (88).

The observation that NK cells accumulate, proliferate,

and become activated within virus-infected tissues in animals suggests that NK cells participate in defence against virus infections *in vivo*, including lymphocytic choriomeningitis virus (LCMV) and murine cytomegalovirus (MCMV) infections (88,163,164). Mice infected with the LCMV express high IFN levels and NK cell activity in affected organs: spleen, peritoneum, liver, lungs, bone marrow (163). Mice, acutely and chronically infected with LCMV, exhibit enhanced NK-mediated resistance against transplanted tumor cells, suggesting the virus activates NK cell functions. NK cell-deficient mice, such as *beige* mice, are more susceptible to MCMV infections than NK replete mice. Experimentally depleting the NK cell activity in mice by cyclophosphamide, hydrocortisone, or anti-NK1.1 antibodies reduces the animals' resistance against the virus. Transplanting NK cells into the deficient animals restores resistance to MCMV. Welsh et al have found that *scid* mice, who lack mature T and B lymphocytes, demonstrate augmented NK cell activity against LCMV or MCMV *in vivo* infections (164). Finally, Bukowski has found that LAK cells protect mice from MCMV, vaccinia virus and herpes-simplex 1 virus (reviewed in 88). The effector is predominantly the $NK1.1^{+}$, $Lyt\text{-}2^{-}$ NK cell, with a small proportion of $NK1.1^{-}$, $Lyt\text{-}2^{+}$ T cells involved. NK cells similarly offer some protection to mice against

the murine hepatitis virus, Friend virus, encephalomyocarditis virus, and the influenza virus (163).

However, NK cell activity does not account fully for immune protection against viruses. While NK-deficient mice are more susceptible to MCMV infections, these mice are not more susceptible to LCMV infections than normal mice (163). Immune deficient scid mice infected with LCMV or MCMV, although they express increased NK cell numbers and activity, fail to effectively clear either virus and either die from the infection, or develop long-term persistent infections (164). These observations indicate that while the NK cell participates in the response to LCMV or MCMV infections, the lack of mature T and B lymphocytes leads to a failure to resolve the infections. In scid mice, the NK cells are capable of actively limiting the replication and synthesis of the infecting viruses and the NK-mediated viral resistance occurs independent of T or B cell responses. Furthermore, lack of NK cells follows with a more rapid virus-induced death. However, the NK cell is not sufficient in itself to eliminate LCMV or MCMV from the host.

NK cell participation in human virus infections is less clear than in mice. Biron described a patient who expressed normal cellular and humoral immune functions, but completely lacked $CD16^+$, $NKH1^+$ NK cells. The

patient suffered from severe varicella virus and cytomegalovirus infections, indicating that the NK cell may be an important defence against virus infections (88,163). Other humans with similar selective and absolute NK cell deficiencies are frequently infected with varicella zoster virus, cytomegalovirus, and Epstein-Barr virus (153). On the other hand, the NK deficient Chediak-Higashi Syndrome patient experiences normal infections with certain viruses, such as measles virus. Additionally, most immune-deficient individuals who suffer with exacerbated and severe viral infections, express deficiencies in their T or B cell functions rather than deficiencies in NK cell functions (126). The role of NK cells in human virus infections remains uncertain and since NK cells appear to participate minimally in animal virus infection, it seems unlikely that their role in human infections would be prominent.

8.4. Cytotoxicity Against Other Microbial Parasites

While not major mediators in immune defence against bacteria or parasites, NK cells may participate in suppression and destruction of certain pathogenic bacteria and parasites. Investigators have demonstrated that NK cells lyse bacteria-infected cells, fungi and parasites *in vitro*. NK cells lyse HeLa cells infected with the bacteria *Shigella flexneri* (153). Blanchard and Djeu

have found that $CD16^+$, $CD3^-$ NK cells lyse human monocytes infected with Legionella pneumophila (reviewed in 88). Murine and human NK cells bind to and inhibit the growth of some fungi, such as: Cryptococcus neoformans, Coccidioides immitis, and Paracoccidioides brasiliensis (153). C. neoformans is more infectious in beige mice than in normal mice; resistance to the fungus is restored by transplanting normal splenocytes containing intact NK cells into the NK deficient mice. In vitro, NK cells lyse Toxoplasma gondii parasites. Additionally, some parasitic infections involving Toxoplasma and Plasmodium augment in vivo NK cell activity.

8.5. Immunoregulatory Functions

In vitro and in vivo observations demonstrate that NK cells regulate host immune functions, such as hematopoietic regulation and hematopoietic graft rejection. Hematopoietic regulation by NK cells occurs through either direct, cellular contact or through indirect contact by cytokine production (88,107,153). With in vitro assays, NK cells lyse immature bone marrow cells, thymocytes, fetal macrophages, and other hematopoietic stem cells (107,153). $CD16^+$, $NKH1^+$, $CD3^-$ NK cells suppress bone marrow-derived hematopoietic colonies; IFN enhances the suppression (153). NK cell derived supernatants can

influence in vitro hematopoietic colony formation. NK cells secrete colony stimulating factors (CSF), including GM-CSF, and IL-3. Some NK cell populations secrete a factor with colony-inhibiting activity (NK-CIA); it suppresses autologous and allogeneic bone marrow-derived hematopoietic colonies. Since anti-TNFα antibodies inhibit this activity and pure TNFα inhibits colony formation, NK-CIA and TNFα may be related molecules.

Some investigators have observed that LCMV-infected mice which express augmented NK cell activity have marked dysfunctions in their immunological and hematological systems, suggesting that NK cells suppress hematopoietic cells in mice (153). Current studies have demonstrated that NK cells in F_1 hybrid recipient mice, recognize and reject allogeneic and parental bone marrow transplants, an event known as hybrid resistance (88,153). Compared with NK sufficient mice, mice experimentally depleted of NK cells and the beige mutant mouse are less likely to express hybrid resistance. Adoptively transferring NK cell clones into the deficient animals restores hybrid resistance.

NK cells may partially regulate the adaptive immune responses. Some investigators found that $CD16^+$, $CD2^+$ NK cells suppress mitogen-induced polyclonal B lymphocyte differentiation (153). Human and murine bone marrow

carries a natural, HNK-1^+ suppressor of B lymphocytes. Alternatively, Katz has demonstrated that NK cells directly interact with and stimulate human activated B lymphocytes (88). Furthermore, some NK cells secrete B cell growth factor (IL-4), augmenting B lymphocyte activity (107). NK cells secrete immunoregulatory cytokines, including: IFNα, IFNγ, IL-1, IL-2, IL-5, a leukocyte chemotactic factor, and a macrophage activating factor (MAF) (88,107,153).

B. CELL MEDIATED CYTOTOXICITY

1. Introduction

The exact molecular events of NK cell-mediated cytolysis are not known but observations that define the process are rapidly accumulating. The rate at which the NK cell lyses its target cell, although slower than that of CTLs, may occur within minutes to hours under optimal conditions (13). NK cell cytolysis involves three discrete steps. The lytic cycle begins when the NK cell recognizes, and binds to the target cell. The second step involves the target cell's activating, and programming the NK cell to deliver its lethal hit. Cytolysis of the target cell is the final stage (13,166,169). After one round of cytolysis, CTLs can immediately lyse another target, and recycle two or three times (172). However, unless

stimulated with IL-2 or IFN, NK cells cannot recycle (13).

2. NK Cell Binds to Target Cells

To lyse its target cell, the NK cell must recognize, and bind to that particular target. Early studies by Bykovskaja demonstrated that CTLs formed conjugates with their target cells (23,155). NK cells, upon target cell recognition, rapidly and strongly adhere to the target, and the binding results in conjugate formation (13,166,169). NK cell-target cell conjugation: follows first order kinetics; depends on extracellular Mg^{2+}, but not on extracellular Ca^{2+}; does not depend on physiological temperature and can occur at 0^{o} C (6,13,24,144,166,172). Studied with electron microscopy, the NK cell-target cell conjugation site demonstrates extensive interdigitation of the cells' plasma membranes (13,24). Conjugate formation involves microfilaments, including actin (13,166).

The recognition and adhesion molecules involved in NK interactions are not completely known, but many potential candidates have been identified. NK cells lack surface immunoglobulin, lack TCR proteins and lack CD3, so unlike B lymphocytes and CTLs, NK cells do not recognize their targets through surface immunoglobulin or the TCR-CD3 complex (78,88,166). In humans and mice, the Fcγ receptor

(CD16) may be the target recognition receptor for ADCC reactions, because anti-CD16 antibodies inhibit ADCC (125). Ortaldo et al have found a novel NK cell receptor described as pNKR or NK-R1 (reviewed in 69,89). The pNKR receptor is expressed primarily by $CD3^-$ cells and antibodies against pNKR block NK cell binding to and lysis of tumor targets. Other surface molecules on NK cells which may act as recognition receptors include CD56 and NKG2 on human NK cells, and NK-R-P1 on rat NK cells (reviewed in 69,89).

Certain surface molecules, often referred to as accessory molecules, cause the strong adhesion between NK cell and its target. The adhesion molecules increase the NK cell's affinity for its target. They include molecules such as fibronectin and laminin (reviewed in 88). About 80-95% of human NK cells express Leu-CAM or β_2 integrin molecules. Current observations indicate the β_2 integrins (LFA-1 in particular) on NK cells mediate target cell adhesion (113,125,166). Other putative adhesion molecules include CD2, CD54 and CD56 (NKH1). Adherence molecules for anchorage-dependent fibroblast targets may differ: arginine-glycine-aspartic acid (RGD) recognizing receptors appear to be the major adhesion molecules, while CD2 and Leu-CAMs participate secondarily (113).

LFA-3 and I-CAM are surface molecules found on NK cell targets (125,166). LFA-3 on target cells binds CD2 with

high affinity and I-CAM similarly binds to LFA-1 on NK cells. LFA-3 and I-CAM may represent target cell molecules to which the NK cell binds.

3. Delivery of the Lethal Hit

Upon physical interaction with its target, the NK cell releases its cytotoxins onto the target cell. The molecular sequence of events within the NK cell, that translates the initial activation to cytotoxin release, is not completely defined. The target cell's interaction with NK cell induces signals within the effector cell that result in the cytolytic response. Observations indicate that CD2 is involved in activating and programming the NK cell for cytolysis. Stimulating the CD2 molecule enhances the activity of the effector cell, including: target cell binding; cytolysis of NK-sensitive and NK-resistant cells; reorientation of granules and associated organelles; secretion of granule contents (125,166). Hiserodt and Timonen have demonstrated that a 48 kD surface molecule on LGLs and LAK cells may activate the lytic process. Cross-linking the molecule stimulates cells to release BLT esterase, while antibodies against the molecule inhibit NK-mediated cytolysis. Some antigenic cross-reactivity exists between p48 and laminin (reviewed in 88).

In activating NK cells, metabolism of phospho-

inositides, increase in cytosolic Ca^{2+}, and activation of protein kinase C (PKC) may be important signals. Some investigators have demonstrated that phosphoinositide metabolism occurs in activated NK cells (10). Plasma membrane inositol is sequentially phosphorylated to phosphatidylinositol-4,5-bisphosphate (PIP_2). PIP_2 is split into inositol-1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG). IP_3 stimulates the release of Ca^{2+} from intracellular stores and DAG activates PKC (98,114). On binding with the appropriate target cell, rat NK (RNK) leukemia cells produce IP_3 and DAG (10). The quantity of IP_3 and DAG produced is proportional to the rate of target cell cytolysis and the target cell's NK-sensitivity. Similarly, Brahmi has demonstrated that LGL-target cell binding stimulates an increase in the phosphoinositide metabolism in LGLs (reviewed in 88).

While CTLs lyse some targets without extracellular Ca^{2+}, NK cell-mediated cytolysis absolutely depends on Ca^{2+} in the external environment (10,13). In all cell types, secretory functions require Ca^{2+} (59). Treating murine and human NK cells with Ca^{2+} ionophores (which increase cytosolic Ca^{2+}) or with phorbol esters (which activate PKC), elicits release of serine esterases, cytolysin, and natural killer cytotoxic factor (NKCF) from the effectors (166). Activation of human NK cells may

also require functional K^+ channels. Reagents such as tetraethylammonium inhibit K^+ channel functioning and inhibit release of NKCF from NK cells and cytolysis of target cells.

Once activated, the NK cell must transfer its cytotoxins onto the target cell. NK cells secrete soluble cytokines, such as IL-1, IL-2, and IFN. However, the mechanism of secretion of these cytokines is unknown. One compelling model demonstrates that cytotoxins contained within the NK cell's azurophilic granules are exocytosed, and directed onto the target cell (13,59,99,166).

3.1. Non-Granule Associated Cytotoxins

NK cells secrete cytotoxins, such as natural killer cytotoxic factor (NKCF), tumor necrosis factor-alpha (TNFα), leukoregulin, and phospholipase A_2 (PLA_2). These are cytotoxins which have not typically been located within cytoplasmic granules. NKCF is a 20-40 kD soluble factor produced by NK cells on interaction with tumor cells (13,18,24,61,88,166). While the mechanism of NKCF action on target cells is not fully understood, this molecule causes slow-acting DNA fragmentation in susceptible target cells (18,166). NKCF is a distinct cytotoxin but antibodies against TNFα partially inhibit NKCF's cytolytic activity. This information suggests that NKCF may be

mediated by multiple components and one component is related to TNFα (24,166). NKCF appears to act on the target cell through specific receptors, is sensitive to trypsin, does not seem to require Ca^{2+} for activity and has not been located within cytoplasmic granules (18,61,166).

Current observations indicate that NK cells can produce TNFα (13,107,116,165). Purified LGL populations, treated with NK-sensitive targets produce TNFα. LGLs treated with NK-resistant targets do not produce the cytotoxin (116). TNFα, a 17 kD soluble cytokine, is found in LGL-derived supernatants but not found in lytic granules (72). Cytokines that activate NK cells, such as IFN and IL-2 enhance TNFα secretion (116). Paya has demonstrated that $CD3^-$, $CD16^+$ NK cells in response to VSV-infected cells produced TNFα and cytolysis of VSV-infected cells was shown to be inhibited by anti-TNFα antibodies (reviewed in 88). TNFα may not be a major cytotoxin in NK-mediated cytolysis, since some NK-sensitive target cells are insensitive to TNFα (116). In vivo, TNFα induces hemorrhagic necrosis of tumors. In vitro, it has proliferative, cytostatic and cytolytic effects on target cells (61,166). Similar to NKCF, TNFα produces DNA fragmentation in target cells (13,166).

Leukoregulin is secreted by some human PBLs and

cultured human NK cells stimulated with NK-sensitive target cells, autologous tumor cells, mitogens, lectins, and Ca^{2+} ionophores (16,61,145). The 32 kD molecule is not directly cytolytic but has cytostatic effects on the target cell and increases the target's sensitivity to cytolysin. In sensitive target cells, the cytotoxin increases plasma membrane permeability, decreases cell volume and changes the cell surface conformation. Leukoregulin appears to induce transmembrane ion channels in the target membrane. The channels are physiologically distinct from those produced by cytolysin. Leukoregulin has not been found in NK cell lytic granules (145,166).

Phospholipase A_2 (PLA_2), a Ca^{2+}-dependent, plasma membrane-associated enzyme, is a putative cytotoxin (13,166). PLA_2 converts the phosphatidylcholine of target cell membranes into lysophosphatidylcholine, a detergent-like cytotoxin. The evidence for PLA_2's cytolytic action is uncertain but PLA_2 inhibitors, such as quinacrine and para-bromophenacyl bromide inhibit NK cell-mediated cytolysis (166).

3.2. Granule Associated Cytotoxins - Cytolysin

Existence of a pore-forming cytotoxin in NK cell granules was first proposed by Henkart et al. They demonstrated that rat LGL leukemia (RNK)-derived granules

contained hemolytic activity similar to that of complement (59). This activity purified to a single, 60-70 kD polypeptide with an acidic pI (13,24,170). The isolated protein, cytolysin, is related to CTL granule-associated perforins and C9 of complement. Cytolysin, perforin, and C9 are functionally and structurally homologous molecules; the homology occurs primarily within the molecules' pore-forming regions (84,88,148,155). Cytolysin is neither associated with non-cytolytic cells nor with cytolytic macrophages and granulocytes (24,155). Within the NK cell, cytolysin does not occur in any subcellular location outside of the cytoplasmic granules (13,166,170).

Cytolysin is a true cytotoxin. Purified cytolysin has potent hemolytic activity and can lyse RBCs within 10 minutes at 37^{o} C (24,59,99,166,170). Cytolysin's hemolytic activity depends on temperature, pH, and Ca^{2+}. Optimal hemolysis occurs at 37^{o} C, occurs more slowly at room temperature and does not occur at 0^{o} C. Hemolysis occurs only at physiological pH and is lost at pH below 6. Cytolysin absolutely depends on Ca^{2+} in the extracellular medium. Extracellular Ca^{2+}-chelating agents such as EGTA inhibit hemolytic activity; hemolysis is restored when Ca^{2+} is returned to the medium (59,99,166,170).

In the same manner as intact NK cells and granules, cytolysin lyses nucleated cells, including: YAC-1 lymphoma

cells, J44 macrophages, K562 cells, EL-4 cells, and NIH 3T3 fibroblasts (99,166). Lysis of nucleated cells similarly requires 37^{o} C, physiological pH and extracellular Ca^{2+}. However, in contrast to RBCs, larger doses of cytolysin are required to effectively lyse nucleated cells. Nucleated cells are known to repair cytolysin-induced damage, a process absent in RBCs (11,59,112,166).

Several investigators have found that cytolysin primarily causes membrane damage in nucleated target cells. Cytolysin induces ^{51}Cr release, which generally indicates membrane damage, from YAC-1 target cells (59,118,144). However, cytolysin cannot induce the release of ^{3}H-thymidine- or ^{125}I-IUdR-labelled DNA from YAC-1 cells, indicating that cytolysin does not produce DNA fragmentation in sensitive target cells (118,119,155).

Sublethal quantities of cytolysin depolarize the target cell's plasma membrane, suggesting that membrane perturbations may be the first event in cell damage. In lipid bilayers, cytolysin causes membrane electrical resistance changes indicating ion channels are incorporated into the bilayer (166,169). Cytolysin induces the release of several small molecular markers from RBC ghosts, lipid vesicles, and target cells. The membranes become leaky to small molecules and ions, such as carboxyfluorescein, Lucifer yellow, sucrose, K^{+}, Na^{+}, Li^{+}, Cl^{-}, Ca^{2+},

Mg^{2+}, Zn^{2+} and Ba^{2+} (13,166,169).

Electron microscopic studies have generated more direct evidence in favour of cytolysin-induced membrane damage. The membranes of cytolysin-treated RBCs, lipid vesicles, and nucleated cells contain protein-lined, circular transmembrane lesions 15-17 nm in diameter (13,59,99,166). The lesions resemble those caused by C9 of complement, perforin from CTLs, and other pore-forming molecules (13,112,155,166). Similar to cytolysin-induced cytolysis, this pore-formation requires 37^{o} C, physiological pH, and extracellular Ca^{2+} (13,59,99,166).

The size of the transmembrane lesions visualized in the target cells indicates that, within the membrane, cytolysin forms a multiunit aggregate (155,166,169). Additionally, in the presence of Ca^{2+}, cytolysin has a molecular weight of 10^{3} kD suggesting the polymerization of individual molecules. Polymerization requires physiological temperature (above 30^{o} C) and Ca^{2+}. The polymers, once formed, are extremely resistant to high temperatures, detergents and disulfide reducing agents. An interesting property of cytolysin is it damages target cells only when it is presented in a monomeric form; presented to cells in the polymerized form, produced by Ca^{2+} and temperature, it no longer induces transmembrane ion channels in the target cell (166).

The evidence presented offers a hypothetical model by which cytolysin acts on and damages the target cell. On interaction with the target cell membrane, cytolysin monomers initially bind to the membrane. Cytolysin does not interact with target cells through specific cell surface receptors, but binds directly to the membrane phospholipids, most likely phosphatidylcholine residues (155). In the presence of extracellular Ca^{2+} and physiological temperature, the membrane-bound cytolysin molecules polymerize into transmembrane tubular structures, creating large voltage-resistant, non-specific ion channels (13,59,88,166).

These ion channels lead to the demise of the target cell. Most nucleated cells are more resistant than RBCs to cytolysin-induced damage and some nucleated cells remain completely unharmed by cytolysin (11,63,85,166). Lettre tumor cells are insensitive to lethal concentrations of cytolysin; the cells lose little intracellular lactate dehydrogenase, and regain their permeability barrier within minutes (11). Cytolysin does not efficiently lyse some nucleated cells, because these cells may have some capacity to repair the damage. Nucleated cells may eliminate transmembrane channels by exocytosing or endocytosing the affected membrane. Alternatively, if the target cell has not been completely depolarized, trans-

membrane channels may be closed by a voltage-driven mechanism (11,112). Neutrophils and U937 cells eliminate complement-induced ion channels from membranes (112,121). Finally, some investigators suggest that a soluble homologous restriction factor inhibits cytolysin activity on target cells (173). However, there is not much support for this hypothesis.

3.3. Granule Associated Cytotoxins - Serine Esterases

Serine esterases in CTL granules have been thoroughly characterized and current evidence indicates that NK cell granules are also enriched in serine esterase activity (13,166). Unlike the granules of macrophages and granulocytes, NK cells do not express non-specific proteases. Macrophages and granulocytes lack serine esterases (99,129).

Several investigators have demonstrated that serine esterases exist in NK cell granules. The granules of human and rodent NK cells express carboxypeptidase and tryptic serine esterase activity (68). Masson and Tschopp have isolated eight unique serine esterases, termed granzymes, from murine CTL granules (155,166). All eight granzymes have not yet been found in NK cells but several related species have been isolated from NK granules. Gershenfeld et al have isolated a trypsin-like granzyme

from $CD16^+$, $CD3^-$ NK cells, while Trapani's group has isolated a granzyme from IL-2 stimulated PBLs (44,45,151). Current observations have additionally found that NK cell granules contain a molecule that chemically reacts with radiolabelled diiodopropylfluorophosphate (DFP), an agent that specifically binds serine esterases (166).

Other investigations have demonstrated that serine esterases participate in NK cell-mediated cytolysis. The macromolecular antiproteases, alpha-1-antitrypsin and alpha-1-antichymotrypsin, block human NK cell-mediated cytolysis (128). DFP and phenyl-methylsulfonylfluoride (PMSF) specifically inhibit serine esterase activity; the reagents block cytolysis by NK cells (128,166). A reagent from organophosphorous pesticides, O,S,S-trimethyl-phosphorodithioate (OSS-TMP) is a potent serine esterase inhibitor that preferentially blocks carboxypeptidase activity. Target cell cytolysis by human NK cells is likewise blocked by OSS-TMP and the inhibition occurs during and/or after the recognition and adhesion step, prior to Ca^{2+}-dependent programming for lysis. This observation suggests that NK-mediated cytolysis requires the action of serine-dependent proteases that specifically cleave aromatic amino acid residues.

Serine esterase activity has some action in the NK lytic process but it remains less obvious how the enzymes

participate in the process. Isolated serine esterases, by themselves, do not appear to lyse target cells, so they may not act as direct cytotoxins (151). There seems to be an exception to this observation: Hayes et al have found that isolated CTL granule-associated granzyme A, in the presence of cytolysin, produces DNA fragmentation and cytolysis in EL-4 target cells (56). Aside from this possible cytotoxic role for serine esterase activity, another model suggests that serine esterases may participate in delivering the lethal hit by proteolytically activating granule-associated cytotoxins (24,44,45,128,166).

3.4. Granule Associated Cytotoxins - Others

In addition to cytolysin and serine esterases, NK cell granules contain proteoglycans; granule-associated proteoglycans are very large macromolecules (200-250 kD), negatively charged and protease-resistant (68,139,166). Through X-ray dispersive analysis, proteoglycans, particularly chondroitan-sulphate A, have been located within NK cell lytic granules.

NK cells release proteoglycans during their activation, and during target cell cytolysis. Stimulating secretory processes in NK cells promotes the release of chondroitan-sulfate A proteoglycan; by contrast, inhibiting secretion in NK cells blocks proteoglycan release

(139,166). The quantity of chondroitan-sulfate A released by stimulated NK cells is directly proportional to the rate of target cell cytolysis (68,139). The function of granule-associated proteoglycans in the cytolytic process remains unknown. Investigators have hypothesized that the molecular complex acts as a substratum on which other granule molecules anchor themselves. These macromolecules resist protease activity, and have a large negative charge, suggesting that they may complex with the basically charged serine esterases and prevent autolysis of the NK cell (68). Extracting cytolysin activity from NK cell granules requires high salt concentrations (1-2 M NaCl) and ion-exchange, heparin affinity columns. This indicates that cytolysin likewise may be complexed to the proteoglycans (155,166). The actual function of proteoglycans in the cytolytic step remains unclear.

There is a strong possibility that other, yet unknown cytotoxins exist within NK cell granules. John Ding-E Young's group has demonstrated that CTL granules depleted of perforin activity still lyse target cells. The CTL-associated cytotoxin is a 50-70 kD molecule that produces DNA fragmentation in target cells and shares very limited serological but not structural homology with TNFα (86,166). Similarly, Podack et al observed that NK cell granules mediate DNA fragmentation in nucleated cells

(119). The cytotoxicity although undefined, occurs independent of cytolysin; loss of cytolysin/perforin activity from granules does not abolish the cytotoxicity. The factor lyses its targets within 3-18 hours, similar to the cytotoxin described by Young. Recent experiments by Shi have described a 32 kD molecule from rat NK cell granules which is cytotoxic for YAC-1 lymphoma cells (146). The cytotoxin, named "fragmentin", produces DNA fragmentation and apoptosis in YAC-1 target cells. The DNA fragmenting activity is rapid, occurs within 4 hour, and requires the presence of cytolysin for DNA cleavage in YAC-1 targets. Fragmentin demonstrates no serological or structural cross-reactivity to TNFα and its cytotoxic action cannot be blocked by anti-TNFα antibodies. Fragmentin appears to be a novel granule-associated molecule and is not related to BLT esterase activity.

3.5. Exocytosis of Granule Associated Cytotoxins

NK cells possess cytotoxins, such as NKCF, TNFα, and leukoregulin, which have not been identified within lytic granules. However, the presence of cytoplasmic granules containing potent cytotoxins and accessory molecules indicates that granules are important participants in the cytotoxic process (13,59,99,117). The granule exocytosis model states that when NK cells adhere to their target

cells, the NK granules reorient themselves and migrate towards the site of NK cell-target cell conjugation. The granule contents or whole granules, are exocytosed into the intercellular cleft between conjugated cells, where granule molecules are rearranged and directed onto the target's plasma membrane (13,24,59,166). The biochemical triggers for granule exocytosis have not been determined, but as described previously, metabolism of phosphoinositides, increases in cytoplasmic Ca^{2+} and activation of protein kinase C may be involved (10,98,114).

Granule exocytosis is a secretory process and studies have demonstrated that reagents that inhibit cellular secretion inhibit NK-mediated cytotoxicity (59). Monensin blocks cellular secretion. Lysosomotropic reagents, such as chloroquine increase the pH of lysosomes and impair both lysosomal functioning and secretion. Cytochalasin B disrupts microfilaments and colchicine disrupts microtubules; both reagents inhibit secretion. All reagents block NK cell-mediated cytolysis (13,24,113,155,166). Cl^- ions in the extracellular milieu are necessary for cellular secretion. Replacing Cl^- with other ions or treating the effector cells with Cl^- channel blockers, such as stilbene disulfonate derivatives, inhibits secretion and NK cell-mediated cytolysis (166).

Electron microscopic and cinemicrographic studies

have demonstrated that granules and Golgi stacks reorient and accumulate at the site where NK cell conjugates with its target. Granule material has been visualized within the intercellular space between conjugated NK and target cells. Furthermore, actin, talin, and the microtubule organizing centre (MTOC) but not myosin, accumulate within this contact region (13,24,99,155,166). In CTLs, microtubule stabilizing agents, such as deuterium oxide and hexylene glycol, inhibit reorientation of granules and associated materials and subsequently inhibit cell-mediated cytolysis (166).

Treating NK cells with Sr^{2+} depletes the cytoplasmic granules: that is, it degranulates the cells. Sr^{2+}-treated NK cells experience a temporary loss of cytotoxicity (13,59). Furthermore, after one round of target cell cytolysis, the NK cell, unless stimulated with IL-2 or IFN, similarly experiences temporary inactivation of its cytotoxic potential. The loss of cytotoxicity suggests that functional secretion is the mechanism of the lytic hit (13).

Human patients with Chediak-Higashi Syndrome (CSH), and mice homozygous for the beige mutation express a genetic defect in which lysosomal function and secretion are impaired (13,24,53,127). In both cases, the defect impairs the lytic granules of lymphocytes, including NK

cells; the cytolytic cells cannot degranulate (24). In CSH patients and in beige mice NK cell cytotoxic activity is reduced or completely absent (13,24,127).

4. Target Cell Cytolysis

Once the lethal molecules are delivered onto the target cell, the NK cell dissociates itself from the target and cytolysis ensues. The biochemical events occurring within the target cell during cytolysis are not clear but the target may undergo several forms of damage. As measured by ^{51}Cr release assays, the target cell may experience membrane damage; intact NK cells, NK granules, and purified cytolysin induce ^{51}Cr release from some target cells (59,99,144). Measured by the release of ^{125}I-IUdR-labelled DNA, target cells may experience DNA damage when exposed to intact NK cells and granules, but this is not produced by cytolysin alone (29,38,119,146). An early and still valid explanation for target cell cytolysis states the target cell experiences colloid osmotic lysis resulting from membrane permeability and cellular ion changes (13,119,166). Alternatively, pore-formation and ion flux changes may result in target cell necrosis, rather than osmotic lysis (75,126,175). Both forms of damage result in the extensive fragmentation of the cells' plasma membrane. Since pore-formation and its

results do not adequately explain DNA degradation, another model of cytolysis involves an autocatalytic cascade of events, leading to the internal disintegration of the target cell which brings about cell lysis. The signals for internal disintegration of the target cell are not completely understood (13,119,133,166).

4.1. Target Cell Cytolysis - Cytolysis Induced by Transmembrane Pore Formation

In 1968, Rosenau demonstrated that upon contact with lymphocytes the target cell swells and eventually ruptures; the swelling was found to be the result of water flowing into the target cell (166). Henney demonstrated that after contact with lymphocytes the target cell develops a new diffusion limited leakage pathway, causing the loss of intracellular electrolytes and molecules. He observed that affected target cells initially release small molecular markers, such as ATP and the K^+ analogue $^{86}Rb^+$, indicating the presence of discrete transmembrane lesions. He observed the subsequent release of larger molecular markers such as ^{51}Cr-labelled proteins, ^{3}H-thymidine- or ^{125}I-IUdR-labelled DNA. Release of the larger molecules could be inhibited by addition of large molecular weight dextrans to the extracellular medium. The dextrans effectively "balanced" the macromolecules

within the target cell, prevented osmotic pressure changes in the target and inhibited cytolysis. Henney proposed that during the time of ion flux changes, water must enter the target cell and cytolysis occurs from excess water intake or colloid osmotic lysis (6,166,169).

Intact NK cells, granules and cytolysin induce large voltage-resistant transmembrane pores in the target cell membrane (166). The transmembrane ion channels change the target's membrane permeability barrier, causing ions to flow into and out of the cell, down their respective electrochemical gradients. As described previously, investigators have observed this membrane permeability to ions and small molecules. K^+ flows out of the cell and Na^+ and Ca^{2+} flow into the cell, thus changing the ion composition of the cell without changing the macro-molecular composition. This may alter the osmotic pressure gradient between the target cell and its environment, water enters the cell, eventually causing the cell to rupture, with extensive membrane damage (13,59,99,166).

Pore formation may cause physiological effects in the target cell other than simple osmotic lysis. For example, pore formation may induce necrosis in cells. Necrosis seems to absolutely depend upon Ca^{2+} and lysis occurs as a result of increases in the target cells' intracellular Ca^{2+} concentration (75). Upon pore-formation, Ca^{2+} enters

the target cell. Across the plasma membrane of cells an extremely large Ca^{2+} gradient exists (10,000 fold difference) (126). With pore formation, a substantial quantity of Ca^{2+} can enter into the target cell and the result depends on the cell's ability to pump out the invading ions (112). Small, transient increases in intracellular Ca^{2+} may be relieved by the cell's repair mechanisms: either by elimination of the ion channels or by pumping out the excess ions via the Na^{+}/Ca^{2+} antiport (11,75). The importance of the Na^{+}/Ca^{2+} exchange has been illustrated by Kraut et al. Inhibitors of the Na^{+}/Ca^{2+} antiport, such as ouabain or 2',4'-dimethylbenzamil, cause the cytolysis of YAC-1 cells by quantities of cytolysin normally too low to cause cellular damage (75). Additionally, small increases in intracellular Ca^{2+} may stimulate cellular repair mechanisms. If the Ca^{2+} increase is large and sustained, irreversible necrosis can occur and includes: membrane damage and membrane blebbing; mitochondrial damage and energy exhaustion; cytoskeleton damage and possibly DNA damage. Additionally, pores may allow other cytotoxins access into the target cell, either directly, or through enhanced endocytosis (119).

4.2. Target Cell Cytolysis - Internal Disintegration Model

While cytolysin appears to cause pore-mediated damage

in susceptible target cells (as described above), the NK cell induces rapid and extensive DNA fragmentation in other targets, an effect that cannot be fully explained by cytolysin and colloid osmotic lysis or necrosis (29,38,119,146,166). Other observations further indicate that another mechanism of cytolysis is occurring in some target cells. The DNA damage often precedes substantial ^{51}Cr release or membrane damage (166), suggesting that DNA degradation may occur before osmotic lysis or necrosis can cause substantial cell damage. Furthermore, DNA degradation results in release of oligonucleosome-sized fragments, which indicates the action of an endonuclease, rather than damage inflicted by excess water uptake by the cell (119,146,166,175). Cytolysin, a pore-forming cytotoxin, is incapable of producing DNA fragmentation in cells (119,166).

Investigators have described a form of cell damage, known as apoptosis or the internal disintegration of the cell (13,36,166). This damage seems to be similar to the process of natural cell death that occurs after exponential growth, and resembles the cytolysis of immature thymocytes after exposure to glucocorticoids. Apoptosis appears to represent cellular self-destruction that has been switched on in response to an environmental stimulus (166). The target cell undergoing apoptosis has the following

physical characteristics: zeiosis, or membrane blebbing; detachment of adherent cells from their substratum; chromatin condensation and DNA fragmentation (36,91,157). On contact with the effector signal the target cell experiences membrane derangements. Membrane depolarization initiates K^+, Na^+, Ca^{2+} fluxes and subsequently may lead to a rise in intracellular Ca^{2+} (4,5,13,96,97). If ionic derangements are small and transient, the target cell may overcome the disturbance and return to normal. However, if derangements are large and persistent, the target cell depletes its energy resources, cannot maintain K^+, Na^+, and Ca^{2+} gradients and autolytic apoptosis may ensue (13,126,166). Apoptosis can occur in certain target cells after contact with glucocorticoids (157), CTLs (36,91), TNFα (91), and NK cells (91,146). However, CTL-derived perforin and NK cell-derived cytolysin are incapable of inducing internal disintegration in cells (4,146).

The target cell may undergo membrane damage in the apoptotic process (13,67,91,166). The target cell membrane undergoes zeiosis or violent membrane "boiling", and adherent cells, such as fibroblasts may become detached from their substrate (91). Russell et al have demonstrated that CTLs induce loss of adhesion in anchorage-dependent L929 cells (133) and TNFα similarly produces loss of adherence in L929 targets (95). Complement plus antibodies

cannot replicate this cellular effect (133) indicating that pore-formation is not sufficient to produce this loss of adherence.

With the internal disintegration mechanism of target cell cytolysis, chromatin condensation and DNA fragmentation are primary and consistent effects (13,36,91,97,146). As indicated earlier, the DNA damage is an early event preceding significant membrane damage. The target cell's DNA is cleaved into soluble, repeating oligonucleosome-sized fragments of 150-180 base pair units, indicating the action of an endonuclease. It is unclear whether the endonuclease is an effector cell-associated cytotoxin or whether endonuclease activity, residing within the target cell, is stimulated by the apoptotic signal (36,37,91,166).

The trigger for apoptosis, whether from CTLs, NK cells, glucocorticoids, or TNFα, has not yet been identified. CTL- and NK cell-mediated apoptosis may involve direct physical interaction of the target with effector cell or may be the result of an effector cell-associated cytotoxin (13,166). NK cell granule-associated fragmentin induces apoptosis in YAC-1 cells (146).

Internal disintegration depends on an early and sustained increase in intracellular Ca^{2+} within the target cell (5,13,67,96,97). Increases in intracellular Ca^{2+} has

multiple effects in the target cell: it stimulates cellular phospholipases that may produce plasma membrane damage; it damages mitochondria and cellular ionic pumps, leading to energy depletion; it induces cytoskeletal changes that may result in zeiosis and loss of substratum adhesion; it activates Topoisomerase II which uncoils DNA; it stimulates endonucleases that fragment target cell DNA (5,13,75,96,97,126). Finally, there is some evidence that the maturity and proliferative capacity of the target cell influences its susceptibility to the apoptosis process. Immature, proliferative thymocytes are susceptible to apoptosis, whereas mature thymocytes remain resistant to the process (97).

C. BASIC FIBROBLAST GROWTH FACTOR

Basic fibroblast growth factor (bFGF) was discovered through its capacity to stimulate proliferation in and phenotypically transform BALB/c and 3T3 fibroblasts (48,49). Basic-FGF is a 16 kD, single chain polypeptide, produced by multiple tissues. Basic-FGF is expressed during the embryogenesis of vertebrates (47). Basic-FGF mRNA and protein have been detected in mouse and chick embryonic tissues and amphibian blastula-stage embryos. Basic-FGF also occurs in adult mammalian and terminally

differentiated tissues. It is a multifunctional mitogen, a growth and differentiation factor for numerous mesodermal and neuroectodermal cells, such as fibroblasts, vascular smooth muscle cells, myoblasts, melanocytes and glial cells (19,47,48,49). The growth factor belongs to an FGF family, which includes: acidic FGF; the int-2 gene family; the hst/K-FGF gene product; FGF-5; FGF-6; keratinocyte growth factor (47,80). All members of the FGF family, while variable in size and amino acid sequence, share a highly homologous core of 120 amino acid residues (47).

Basic-FGF is a fundamental regulatory molecule for the control of cellular growth and differentiation as well as possessing angiogenic properties, participating in wound healing and inducing ectodermal tissues into mesoderm (47,48). It acts through autocrine and paracrine mechanisms (48,49). With bFGF-stimulation, responsive cells, such as BHK-2 and NIH 3T3 cells, develop a transformation-like phenotype that includes: reduced substratum adherence, increased membrane ruffling, and in some cases, growth in soft agar (49). Other morphological changes, and random cell migrations are evident. Basic-FGF is a potent stimulator of cellular proliferation and DNA synthesis (22,47,48). In a given time span, bFGF-treated cells go through more cell doublings than bFGF-deprived, quiescent cells. Finally, bFGF delays the ultimate

senescence of cultured cells (48,49).

While the molecular mechanism of bFGF action within target cells remains unknown, the growth factor induces expression and synthesis of certain proteins (19,48,49). Basic-FGF stimulates synthesis of thiol-dependent cathepsin and plasminogen activator as well as synthesis of the extracellular matrix proteins collagen, fibronectin, laminin, and proteoglycans (47,49). The growth factor augments expression of the cellular oncogenes, c-myc and c-fos, oncogenes that regulate cellular differentiation and proliferation (49). Furthermore, bFGF acts on the cytoskeleton of responsive cells and produces such effects as inducing rapid changes in cytoskeleton-associated actin.

Basic-FGF is an unusual growth factor in that it is not conventionally secreted by producing cells (48,49). It is unclear how it is secreted but bFGF is released and directly incorporated into the extracellular matrix, becoming complexed with the extracellular matrix molecules, in particular with heparin or heparan-sulfate proteoglycans (47,49). However bFGF is secreted, it acts on responsive cells through specific and high affinity cell surface receptors (48,49,80,103). The bFGF receptor is a transmembrane complex consisting of two components: a 145 kD molecule that preferentially binds bFGF; a 125 kD molecule that preferentially binds aFGF. The receptor's

intracellular domain has tyrosine kinase activity (80,103). Trypsin digestion of the receptor yields four peptides which carry a consensus sequence for tyrosine kinase. Tyrosine kinase activity may be responsible for target cell stimulation (62,103). The bFGF receptor is homologous to the receptors for platelet derived growth factor and colony stimulating factor-1, particularly within the tyrosine kinase domains (80). In addition to these high affinity receptors, bFGF also binds to cells and the extracellular matrix at low affinity sites (47). These sites correspond to cell surface-associated heparan-sulfate proteoglycans and binding occurs independent of bFGF-receptor associations.

It remains unclear whether bFGF acts at the extracellular receptor site or at an intracellular site, such as the nucleus. Upon receptor binding, the bFGF-receptor complex is endocytosed into the cell and has been found to be translocated to the nucleus, one putative site of bFGF action (103). Basic-FGF translocation to the nucleolus has been positively correlated with stimulation of ribosomal gene transcription during the G_o to G_1 transition in the cell cycle (19). Basic-FGF has similarly been identified within the nucleolus of aortic endothelial cells (103). It is unclear how bFGF interacts with the cell's nucleus to bring about its mitogenic effects.

CHAPTER 3: INTRODUCTION

Although the exact molecular events of CTL- or NK cell-mediated cytolysis remain unclear, cytolysis involves either target cell membrane damage (59,144), loss of target cell's adherence to its substratum (1,133) or DNA fragmentation (29,118,146,175). CTL- or NK cell-associated perforin/cytolysin creates transmembrane ion channels in the target cell membrane, eventually causing membrane damage and cytolysis of the target cell (59,166). Another form of CTL-induced target cell damage, described by Russell et al, is loss of the target cell's adherence to its substratum. Loss of adherence is specifically caused by CTLs but not by complement, suggesting that pore formation is not sufficient to induce this damage. Furthermore, the causative agent in CTLs remains unknown (1,133). TNFα, a cytotoxin secreted by cells such as lymphocytes and macrophages, also produces loss of adherence in L929 fibroblasts (94,95). Loss of adhesion is lethal for the detached cells but it remains unclear whether cell death occurs before, or as a result of detachment. CTLs, NK cells and their granules as well as TNFα, induce DNA fragmentation in target cells (29,95,118,146). DNA fragmentation occurs independently of perforin or cytolysin activity for some target cells but absolutely requires it with other target cells (146). The mechanism

by which CTLs, NK cells or TNFα induce DNA fragmentation remains unclear.

NK cells lyse undifferentiated cells, such as thymocytes, embryonic macrophages, hematopoietic stem cells, and immature fibroblasts. The undifferentiated cells are more susceptible to cytolysis than differentiated cells (107,153). Immature fibroblasts are more responsive to the effects of growth factors such as bFGF, and respond with a high rate of proliferation. Growth factor responsiveness and proliferation disappear with increasing cellular maturity (49,62,72). Khalil previously demonstrated that NK cells preferentially lyse growth factor-stimulated, actively proliferating 10T1/2 fibroblasts (72). When deprived of certain serum factors, 10T1/2 fibroblasts enter a quiescent or growth-inhibited state, and remain fairly resistant to cytolysis by intact NK cells. Inducing proliferation in 10T1/2 with bFGF increases their susceptibility to NK-mediated cytolysis. The growth factor-enhanced NK sensitivity parallels the rate of target cell proliferation and depends on the dose of bFGF and the time target cells are exposed to bFGF. Finally, cold target inhibition experiments established that bFGF does not augment the NK cell's recognition of, and binding to 10T1/2 cells, indicating bFGF-induced NK sensitivity occurs at a post-binding cytolytic step.

NK cell cytoplasmic granules represent the cells' post-binding cytolytic apparatus, demonstrate the same lytic potential of the NK cell and may participate in the growth factor augmented cytolysis of 10T1/2 fibroblasts. The experiments in this report were set to test the hypothesis that 10T1/2 fibroblasts are susceptible to cytolysis by NK cell-associated granules, and to test whether the preferential lysis of bFGF-stimulated, proliferating 10T1/2 fibroblasts occurs as a post-binding, granule-associated lytic event. Do NK cell granules lyse 10T1/2 and does bFGF modify the ability of NK cell granule effector molecules to lyse 10T1/2 fibroblasts?

To test this hypothesis, 10T1/2 fibroblasts, quiescent and growth factor stimulated, were exposed to granule cytotoxins isolated from the RNK-16 leukemia cell line. 10T1/2 cells were made quiescent or growth inhibited through incubation in serum-free, bFGF-free defined medium. Cellular proliferation was re-initiated by the addition of bFGF to the quiescent cell cultures. NK cell granule-mediated cytolysis of 10T1/2 was assessed by: (1) ^{51}Cr release assays to measure membrane damage; (2) the cytotoxicity adherence assay to detect loss of 10T1/2 cell adhesion properties; (3) ^{125}I-IUdR release assays to detect DNA fragmentation. Additionally, in order to determine whether cytolysin participates in 10T1/2 cyto-

lysis and bFGF-augmented cytolytic events, 10T1/2 fibroblasts were exposed to heparin-agarose purified cytolysin, or to granule preparations depleted of cytolysin activity.

CHAPTER 4: MATERIALS AND METHODS

1. Cells and Cell Maintenance

1.1. 10T1/2

10T1/2 fibroblasts are a cell line derived from C3H/HeN mouse embryonic cells (123). 10T1/2 were cultured on 10 cm Falcon plastic tissue culture plates (Becton Dickinson & Co., Lincoln Park, NJ) in F12 or alpha-MEM medium containing: 50 units/ml penicillin G, 50 µg/ml streptomycin sulfate, and 10% fetal calf serum (above from: GIBCO Laboratories, Grand Island, NY). Cells were maintained at 37^{o} C in a humidified atmosphere containing 5% CO_2. At confluence, new cultures were initiated. Fibroblast monolayers were incubated 5 to 10 minutes with 0.05% trypsin-EDTA (GIBCO) and gently agitated to disperse into single cells. To remove excess trypsin, the cells were diluted in medium containing 10% serum and centrifuged 10 minutes at 1000 rpm. Sedimented cells, resuspended in F12 or alpha-MEM containing penicillin-streptomycin and 10% fetal calf serum, were dispersed onto tissue culture plates.

1.2. Growth Factors and Medium Factors

Basic Fibroblast Growth Factor: Bovine recombinant bFGF (Boehringer Mannheim Canada. Ltd., Laval, Quebec) was dissolved in sterile F12 medium containing 1.0 mg/ml bovine serum albumin (BSA - SIGMA Chemical Co., St. Louis, Mo), pH 7. A stock solution of 1.0 ng/µl bFGF was made and was stored at -20° C. Fresh stock solution was made every 3 months.

Insulin: Bovine pancreatic insulin (SIGMA) was dissolved in double distilled water, pH below 2. A stock solution of 5 mg/ml was made and aliquots were stored at -70° C. Fresh stock solution was made every month.

Transferrin: Human transferrin (SIGMA) was dissolved in phosphate buffered saline (PBS), pH 7. A 4 mg/ml stock solution was made and aliquots were stored at -20° C. Fresh stock solution was made every 3 to 4 months.

Epidermal Growth Factor: EGF (Collaborative Research, Belford, Ma) was dissolved in double distilled water, pH 7. A stock solution of 20 µg/ml was made and aliquots were stored at -20° C. Fresh stock solution was made every month.

1.3. Defined Medium

Defined medium (DM) was prepared as follows: F12

medium containing 50 units/ml penicillin G, 50 µg/ml streptomycin sulfate, 20 ng/ml insulin, 40 ng/ml transferrin and 10 ng/ml EGF. The DM was membrane filtered with a 0.22 micron Falcon bottle top filter unit (Becton Dickinson & Co.). Fresh DM was prepared for every assay.

1.4. bFGF Induced Proliferation

10T1/2 cells were grown, trypsinized and washed as previously described. Each 10 cm Falcon tissue culture plate was seeded with $2x10^5$ cells in 6.0 ml DM and incubated at 37° C in 5% CO_2 for 24 hours to make the cells quiescent. To re-establish cellular proliferation and growth, 10T1/2 were incubated with bFGF. After 24 hours in DM, the medium was removed and replaced with 6.0 ml DM containing bFGF, 25 ng/ml medium per $2x10^5$ cells. The cells were incubated at 37° C in 5% CO_2 for 48 hours. Several plates of cells were maintained as the quiescent cell population and were incubated at 37° C in 5% CO_2 for 48 hours in 6.0 ml DM without added bFGF. After the 48 hour incubation, plus or minus bFGF, the cells were used in the experiments.

2. Preparation of Granule Proteins

2.1. Growth of RNK Leukemia Cells

The RNK-16 leukemia cell line (obtained from Dr. C. W. Reynolds of the NCI, Frederick, Md) demonstrates LGL morphology, high natural cytotoxicity and is a good source of lytic granules (59,161). The RNK-16 leukemia cells were *in vivo* passaged in Fischer F344 male rats (Charles River Laboratories, Inc., Kingston, NY) pretreated with 2,6,10,14-tetramethylpentadecane (SIGMA): 1.0 ml injected intraperitoneally 3 to 7 days prior to injection of the RNK cells. 2,6,10,14-tetramethylpentadecane prevents solid tumor development in the rats. Then, $2x10^7$ RNK-16 leukemia cells suspended in Hank's balanced salt solution (GIBCO) were injected intraperitoneally into the rats. After 2 weeks, and no later than 3 weeks, the expanded leukemia cells were harvested with colourless HBSS containing 100 units/ml porcine intestinal heparin (SIGMA).

2.2. Isolation of Granules from RNK Cells

Isolating lytic granules from RNK-16 leukemia cells involves disrupting the cells and separating the granules from other subcellular organelles through Percoll density gradient centrifugation (166). The method used to isolate

granules was developed from that of Millard et al (99). RNK-16 cells, harvested from the rats, were centrifuged for 8 minutes at 800 rpm. The sedimented cells were resuspended in sufficient disruption buffer to bring the suspension to $1x10^8$ cells/ml. Disruption buffer, which lyses the cells, contained: 0.25 M sucrose (Mallinckrodt Inc., Paris, Ky), 4.0 mM EGTA (SIGMA), 10 mM HEPES (GIBCO) and 400 units/ml heparin at pH 7.4. The cell suspension was incubated for 20 minutes at 0^o C with constant stirring in a nitrogen-cavitation chamber under 450 psi pressure. With release of pressure, the suspension was forced out of the chamber and the cells were gently ruptured as they passed through the chamber's valve system.

Enzyme digestion and filtration remove nuclear material from the suspension. The suspension was incubated for 30 minutes at room temperature with 1000 units DNAase I (SIGMA) per ml suspension, in the presence of $MgCl_2$ (J. T. Baker Chemical Co., Phillipsburg, NJ). The suspension was successively filtered through 5 micron, then 3 micron Swinex filter units (Nucleopore Corp., Pleasanton, Ca).

To separate intact granules from other subcellular organelles, the nuclear-free homogenate was centrifuged on a Percoll density gradient. Adjusted Percoll was prepared as follows: 48% (v/v) Percoll (Pharmacia, LKB Biotechnology, Baie D'Urfe, Quebec) in double distilled

water, 0.25 M sucrose, 10 mM HEPES, 4.0 mM EGTA and 3.0 mM HCl (Mallinckrodt) at pH 7.4. In Beckman polycarbonate ultracentrifuge tubes (Beckman Instruments Inc., Fullerton, Ca), 5.0 ml of cell homogenate was layered on top of 19.0 ml adjusted Percoll. With a Beckman L8-70M ultracentrifuge, the tubes were centrifuged in a 70 Ti rotor at 20,000 rpm for 10 minutes without deceleration. From each gradient tube, the bottom 5.0 ml was collected: this volume corresponds with the fractions that contain granule material (99). The collected granule fractions were pooled, and in a Beckman L8-70M ultracentrifuge, the material was centrifuged in a 70 Ti rotor for 16 to 18 hours at 34,000 rpm. The concentrated granule preparation, which appears as a band of white, flocculent material, was collected and stored at -20^{o} C for 1 to 2 days.

The cytolytically active material of the isolated granule preparation must be solubilized with high salt concentrations. The frozen material was thawed, solubilized in 2.0 M NaCl (Mallinckrodt) and frozen at -20^{o} C for 1 to 2 days. To eliminate insoluble debris from the preparation, it was thawed a second time, and in a Beckman L8-70M ultracentrifuge, centrifuged in a 70.1 Ti rotor for one hour at 34,000 rpm. The supernatant was collected and aliquots were stored at -70^{o} C. Since the

preparations were solubilized in 2.0 M NaCl, granules used in the experiments were diluted to normal saline concentrations.

2.3. Calcium and Heat Treatment of Granules

Exposing RNK cell lytic granules to Ca^{2+} and heat destroys cytolysin's lytic activity (166). By treating a quantity of granule material with $CaCl_2$ for one hour at 37^{o} C, cytolysin activity is irreversibly eliminated. A constant quantity of granules (diluted in normal saline containing 10 mM HEPES, pH 7) was incubated for one hour at 37^{o} C with millimolar concentrations of $CaCl_2$ (J.T. Baker Chemical Co.) serially diluted in normal saline containing 10 mM HEPES, pH 7. In the assays, the Ca^{2+}-pretreated granules were administered to target cells. The hemolytic activity of cytolysin in the Ca^{2+}-pretreated granules was measured with the SRBC assay.

2.4. Isolation of Cytolysin from Granules

Purified cytolysin, used in these experiments, was donated by Ricky Kraut and Dr. Lianfa Shi and was purified according to the method of Bashford et al (11). Pooled, solubilized granule preparations were centrifuged at 34,000 rpm for 1-3 hours in a Beckman L8-70M ultra-centrifuge. The supernatant was eluted from a gel

filtration column (11 x 2 cm Ultragel AcA54 [Pharmacia]) using as elution buffer, PBS with: 0.5 mM EDTA (SIGMA), 2.0 M NaCl and 10 mM HEPES. Fractions with lytic activity were further purified by dialyzing against PBS containing: 0.2% polyethylene glycol 4000 (SIGMA) and 0.5 mM EDTA at pH 7.4 and 4^{o} C. The dialysate was loaded onto a 1.5 x 8 cm heparin-agarose affinity column (Pierce Chemical Co., Rockford, IL). Material from stepwise elution with buffers of increasing ionic strength were subjected to SDS-PAGE under non-reducing conditions, and silver stained to confirm homogeneity.

3. ^{51}Cr Release Assay

^{51}Cr binds to the macromolecules within cells, and release of the label into the supernatant is an excellent measure of cellular membrane damage and cytolysis (13,144). After 48 hours culture with or without bFGF, 10T1/2 cells were labelled with ^{51}Cr. 10T1/2 cells were incubated at 37^{o} C for 2 hours with 3-4 ml DM (with or without bFGF) containing 150-300 mCi $Na^{51}CrO_4$ (ICN Biomedicals, Inc., Costa Mesa, Ca) per 10 cm plate. The excess label was removed and the cells were washed twice with HBSS. The labelled cells were trypsinized and centrifuged in medium plus 10% serum for 12 minutes at 1300 rpm. Sedimented cells were resuspended in DM (plus or

minus bFGF), and $1x10^4$ cells were seeded into each well of NUNC flat-bottom microtitre plates (Nunclon-Delta, Denmark). The cells were incubated for 24 hours at 37^{o} C in 5% CO_2, allowing cells to adhere to and form a one-cell-thick layer over the base of each well.

Once the monolayer was established, unbound ^{51}Cr and non-adherent cells were removed and each well was replenished with 50 µl DM (plus or minus bFGF, 4.0 mM $CaCl_2$). The ^{51}Cr labelled cells were incubated with 50 µl of granule or cytolysin serially diluted with normal saline containing 1.0 mM EGTA and 10 mM HEPES, pH 7. In another assay, cells were treated with 50 µl of $CaCl_2$- and heat-pretreated granules. Cells were incubated at 37^{o} C in 5% CO_2 for 1 or 4 hours. The assay was terminated by administering 100 µl cold HBSS to each well. The plates were centrifuged at 1500 rpm for 5 minutes to sediment the detached cells. From each well, 100 µl supernatant was harvested and this represents one-half of ^{51}Cr released by the cells; this is the <u>supernatant</u>. Subsequently, 100 µl of 2.0 M NaOH (Mallinckrodt) was added to each well, mixed with the remaining supernatant and cells, and the total volume was collected. This volume represents one-half of the ^{51}Cr released from cells plus the ^{51}Cr remaining within cells, and is the <u>remainder</u>. For each sample, the counts per minute (cpm) were quantitated in a LKB Wallac 1282

Compugamma and cytotoxicity was calculated as percent specific ^{51}Cr release as follows:

$$(1)\ \%\ ^{51}Cr\ \text{release} = \frac{2 \times \text{cpm supernatant}}{\text{cpm supernatant} + \text{cpm remainder}} \times 100$$

$$(2)\ \%\ \text{specific}\ ^{51}Cr\ \text{release} = 100 \times \frac{\%\ ^{51}Cr\ \text{release (T)} - \%\ ^{51}Cr\ \text{release (S)}}{100 - \%\ ^{51}Cr\ \text{release (S)}}$$

where T = test, and S = spontaneous

4. Cytotoxicity Adhesion Assay

Loss of cells' adherence to their substratum has been reported as a form of immune damage inflicted by CTLs (133). It similarly occurs in TNFα-induced damage of L929 fibroblasts (132). The assay, measuring granule-mediated detachment of 10T1/2 cells from their substratum, was developed from the TNFα assay described by Ruff and Gifford (132), and Matthews and Neale (95).

10T1/2 fibroblasts were grown in the presence or absence of bFGF for 48 hours, as previously described. The monolayers were then incubated 1 to 2 minutes with trypsin-EDTA and centrifuged in medium plus 10% serum for 12 minutes at 1300 rpm. Sedimented cells were

resuspended in DM with or without bFGF and $1x10^4$ cells were seeded into each well of NUNC sterile, flat-bottom microtitre plates. The cultures were incubated at 37^{o} C in 5% CO_2 for 24 hours, allowing the cells to adhere, spread out and form a one-cell-thick layer over the base of each well.

The adherent monolayers were treated with granule preparation. The supernatant and non-adherent cells were removed from the established monolayers, and each well was replenished with 50 µl DM (with or without bFGF) containing 4.0 mM $CaCl_2$. The target cells were treated 50 µl of granules or cytolysin serially diluted with normal saline containing 1.0 mM EGTA and 10 mM HEPES, pH 7. In another assay, target cells were incubated with 50 µl $CaCl_2$- and heat-pretreated granules. After an 18-24 hour incubation at 37^{o} C in 5% CO_2, the cells were stained with 0.5% (w/v) crystal violet (SIGMA) in 80:20 water:methanol (Mallinckrodt). The supernatant and detached cells were gently removed from each well and 50 µl crystal violet was administered to each well. After a brief time at room temperature, excess crystal violet was washed away with water. Only adherent, viable cells will bind crystal violet. The cell-bound stain was measured spectrophotometrically with a Titertek Multiskan MCC/340 (Flow Laboratories, Inc., McLean, Va), at 540 nm wavelength.

The stained cells were measured either dry, or with the crystal violet solubilized with 150 µl Sorensen's buffer: 30 mM citric acid (Fischer Scientific Co., Fairlawn, NJ); 20 mM HCl (Mallinckrodt); 95% ethanol (Consolidated Alcohols Ltd., Toronto, Ontario). Percent 10T1/2 loss of adherence or percent cytotoxicity was calculated as follows:

$$\frac{\text{Background absorbance - Test absorbance}}{\text{Background absorbance}} \times 100$$

The viability of the detached cells was determined at the end of the assay. After 10T1/2 cells were exposed to the granule cytotoxins, the supernatant was collected, pooled and centrifuged for 10 to 15 minutes at 1500 rpm. The sedimented cells were stained with 5-10 µl trypan blue (SIGMA) and counted with a hematocytometer. The percentage of dead cells was calculated as follows:

$$\frac{\text{Number of detached cells stained with trypan blue}}{\text{Total number of detached cells}} \times 100$$

5. ^{125}I-IUdR Release Assay

Cells incubated with the radiolabelled molecule ^{125}I-iododeoxyuridine incorporate the molecule into their

DNA (17). Cells release the isotope into the supernatant only upon cytolysis and some investigators have found that ^{125}I-IUdR release directly corresponds to DNA fragmentation on agarose gel electrophoresis (36,146). 10T1/2 cells were cultured with or without bFGF as previously described. The cells were labelled as follows: cells were incubated at 37^{o} C for 90 minutes in 3-4 ml DM (plus or minus bFGF) containing 0.01-0.02 mCi ^{125}I-IUdR (ICN Biomedicals) per 10 cm plate. To remove excess, unbound ^{125}I-IUdR, the labelled cells were twice washed with HBSS. Cells were trypsinized and centrifuged as described in the adhesion assay, and were resuspended in DM with or without bFGF. Then, 1x10^{4} cells were seeded into each well of NUNC flat-bottom microtitre plates and incubated for 24 hours at 37^{o} C in 5% CO_2 to allow the labelled cells to form an adherent, one-cell-thick layer over the base of each well.

Supernatant containing unbound ^{125}I-IUdR and non-adherent cells was removed and each well was replenished with 50 µl DM (plus or minus bFGF). 10T1/2 monolayers were treated with 50 µl of granule or cytolysin serially diluted with normal saline containing 1.0 mM EGTA and 10 mM HEPES, pH 7. In another assay, cells were incubated with 50 µl $CaCl_2$- and heat-pretreated granules. Cells were incubated at 37^{o} C in 5% CO_2 with granule cytotoxins for 2, 4, or 18 hours. To terminate the assay, 100 µl cold HBSS was added

to each well and the plates were centrifuged for 5 minutes at 1500 rpm to sediment any detached cells. From each well, 100 µl supernatant was collected and this represents one-half of the ^{125}I-IUdR released from the cells; this is the supernatant. Subsequently, 100 µl of 2.0 M NaOH was administered to each well, mixed with the remaining supernatant and cells, and the total volume was collected. This represents one-half of ^{125}I-IUdR released by cells plus the label remaining within unlysed target cells, and is the remainder. Counts per minute (cpm), for each sample, were quantitated with an LKB Wallac 1282 Compugamma, and percent specific ^{125}I-IUdR release, or percent DNA fragmentation was calculated as follows:

(1) % ^{125}I-IUdR release =

$$\frac{2 \times \text{cpm supernatant}}{\text{cpm supernatant} + \text{cpm remainder}} \times 100$$

(2) % specific ^{125}I-IUdR release = 100 x

$$\frac{\%\ ^{125}\text{I-IUdR release (T)} - \%\ ^{125}\text{I-IUdR release (S)}}{100 - \%\ ^{125}\text{I-IUdR release (S)}}$$

where T = test, and S = spontaneous

6. SRBC Assay for Cytolysin Activity

The hemolytic activity of granule-associated cytolysin can be measured in the SRBC assay described by Millard (99). SRBCs (National Biological Laboratories, Dugald, Manitoba) were centrifuged at 2000 rpm for 10 minutes in PBS. Sedimented SRBCs were resuspended to $7x10^7$ cells/ml in normal saline containing: 2.0 mg/ml BSA, 4.0 mM $CaCl_2$, 10 mM HEPES and 0.2% PEG 4000 at pH 7. In each well of Linbro/Titertek, V-bottom microtitre plates (Flow Laboratories), 100 µl of the SRBC suspension was combined with 100 µl of granules or cytolysin serially diluted with normal saline containing 1.0 mM EGTA and 10 mM HEPES, pH 7; or with 100 µl of $CaCl_2$- and heat-pretreated granules. The SRBCs were incubated with cytotoxins at 37^{o} C in 5% CO_2 for 15-30 minutes. To terminate the assay, the plates were centrifuged at 1500 rpm for 5 minutes. From each sample, 150 µl supernatant was harvested and the released hemoglobin was measured spectrophotometrically in a Titertek Multiskan MCC/340 at 414 nm wavelength. Percent hemolysis was calculated as follows:

(Absorbance Test - Absorbance Background) x 100

7. Protein Determination

The protein concentration of isolated granule

preparations was determined with the Bio-Rad protein reagent kit (BioRad Laboratories, Richmond, Ca). A protein standard curve was established using BSA as the standard: 1.0 µg/ml to 25 µg/ml. The best fitting line was calculated using linear regression. Granule material or BSA was diluted to the appropriate concentration in double distilled water. Then, 0.8 ml of diluted BSA or granule preparation was combined with 0.2 ml of filtered, concentrated Bio-Rad reagent. The absorbance of each sample, read within 60 minutes, was measured with a Beckman DU-8 spectrophotometer at 595 nm wavelength. The concentration of granule was determined from the BSA standard curve and was expressed as the mean of 2 or more values.

CHAPTER 5: RESULTS

1. Hemolytic Activity of Granule Preparation and Cytolysin

Granules isolated from RNK leukemia cells possess the same lytic potential of the intact NK cell (59,99). Granule preparations from different rats may vary in the quality and quantity of their protein/cytotoxin content. One granule preparation, which was isolated from the RNK-16 leukemia cell line grown in F344 rats as ascites, solubilized in 2.0 M NaCl and diluted to 0.145 M NaCl, was used in the reported experiments. The preparation used was chosen for its potent cytolytic activity against SRBCs and 10T1/2 fibroblasts. The target cells were exposed to pretty well the same granule effector molecules in all experiments.

Granules isolated from RNK-16 leukemia cells, as described in Methods, demonstrated potent hemolytic activity against SRBCs. Table 1 indicates that 0.82 to 6.57 µg/ml granule protein produced 80-90% cytolysis of 10^7 SRBC/ml. Cytolysin, which was isolated from RNK leukemia cell granules by the method of Bashford (11) and was purified to a single band on SDS-PAGE, exhibited potent hemolytic activity in SRBC assays. From 4.9 to 39 lytic units/ml cytolysin (where 1 lytic unit/ml equals the final cytolysin dilution producing 50% hemolysis) produced 70-90% cytolysis of 10^7 SRBC/ml. Table 1 also indicates

TABLE 1: Effect of Granules and Cytolysin on SRBC

Granules		Cytolysin	
Granules (µg/ml)	Percent hemolysis	Cytolysin (LU/ml)[a]	Percent hemolysis
6.57	87	39	87
3.29	85	19	88
1.64	77	9.8	83
0.82	86	4.9	73

SRBCs were incubated with serially diluted granules, or cytolysin. Hemolysis was measured and the results represent the mean of duplicate values.

[a]Cytolysin is reported in Lytic Units/ml, where one Lytic Unit/ml = final dilution cytolysin producing 50% SRBC hemolysis.

that 4.9 to 39 lytic units/ml of cytolysin produced a proportional rate of hemolysis that 0.82 to 6.57 ug/ml of granules produced.

In order to determine the necessity of cytolysin in granule-mediated 10T1/2 cytolysis, assays were performed with granules which had been depleted of its cytolysin activity. Both Ca^{2+} and physiological temperature have been found to inactivate cytolysin (166). Ca^{2+} and temperature induce the polymerization of cytolysin. This polymerized form, when presented to target cells, can no longer produce transmembrane pores and cell damage. RNK granule material was pre-incubated with $CaCl_2$ at 37^{o} C for one hour, and to determine whether this treatment depleted cytolysin activity, SRBCs were incubated with the $CaCl_2$- and heat-pretreated granules. Table 2 shows that 3.29 µg/ml of granule protein, incubated for one hour at 37^{o} C with 0.2 to 1.6 mM $CaCl_2$, lost its hemolytic, or cytolysin activity against SRBCs. The same quantity of granules incubated at 37^{o} C with lower concentrations of $CaCl_2$, between 0 to 0.1 mM, did not lose cytolysin activity.

While cytolysin can be inactivated by pretreatment with $CaCl_2$ and temperature, cytolysin requires Ca^{2+} and physiological temperature in order to insert into the plasma membrane and polymerize into transmembrane channels,

TABLE 2: Effect of Ca^{2+}-Pretreated Granules on SRBC

$CaCl_2$ pretreatment (mM)	Percent hemolysis
1.6	0
0.8	0
0.4	1
0.2	2
0.1	69
0.05	97
0.02	95
0	96
control[a]	98

SRBCs were incubated with 3.29 µg/ml of granules that were pretreated with $CaCl_2$ for 1 hour at 37° C. Hemolysis was measured and the results represent the mean of duplicate values.

[a]Control represents SRBCs incubated with granule protein which was not pretreated with $CaCl_2$ at 37° C for one hour.

thus producing cellular damage (166). Chelating Ca^{2+} from the assay supernatant with EGTA has been shown to inhibit cytolysin-mediated cell damage (166,168). This technique was used as a second method of eliminating cytolysin activity from the granule preparation. Table 3 illustrates that chelating Ca^{2+} from the extracellular medium with EGTA blocked cytolysin-mediated SRBC hemolysis, while adding excess $CaCl_2$ restored cytolysin activity against SRBCs.

2. bFGF Induced Proliferation in 10T1/2 Cells

Cells deprived of serum growth factors enter into a quiescent state in which growth and DNA synthesis stop. The cells may re-initiate growth and DNA synthesis if provided with appropriate serum factors (28). 10T1/2 fibroblasts, incubated for 24 hours in defined medium (DM) lacking specific serum factors, entered a quiescent state. Incubating the quiescent cells for an additional 48 hours with 25 ng bFGF/ml of DM per $2x10^5$ cells re-initiated cellular proliferation (72). 10T1/2 cells maintained in DM without bFGF for the 48 hour incubation remained quiescent.

To establish whether bFGF induced proliferation and growth in 10T1/2, proliferation in the bFGF-stimulated cell population was compared to that in unstimulated,

TABLE 3: Effect of Extracellular Ca^{2+} on Granule-Mediated SRBC Hemolysis

$CaCl_2$ (mM)	EGTA (mM)	Percent hemolysis
0.7	1.1	1
0.9	1.1	0
1.1	1.1	0
1.3	1.1	3
1.5	1.1	38
1.7	1.1	72

SRBCs were incubated with granules (3.29 µg protein/ml) in assay medium containing 1.1 mM EGTA. From 0.7 to 1.7 mM $CaCl_2$ was added to the cells. Hemolysis was measured and results are expressed as the mean of duplicate values.

quiescent cells by the following techniques. (1) The rate of cell doublings was calculated. As well as stimulating proliferation and DNA synthesis in responsive cells, bFGF greatly reduces the cells' average doubling time (49). With this technique, we compared the number of cells harvested after complete incubation with or without bFGF, to the number of cells seeded at onset of incubation with or without bFGF ($2x10^5$ cells per plate):

$$Z = \log \frac{\text{number cells harvested after incubation}}{\text{number of cells seeded } (2x10^5 \text{ cells/plate})} \Big/ \log 2$$

where Z = number of times the cell population doubled.

The number of cell doublings of bFGF-stimulated 10T1/2 was subsequently compared to the cell doublings of unstimulated 10T1/2 (see table 4). (2) Since ^{125}I-IUdR is readily incorporated into the DNA of actively proliferating cells, but not in non-growing cells, the quantity of the DNA label taken up by 10T1/2 cells should reflect the rate of DNA synthesis. The quantity of ^{125}I-IUdR incorporated into $1x10^4$ 10T1/2 cells after complete incubation with or without bFGF was measured, and the label incorporated into bFGF-stimulated cells was compared to that incorporated into quiescent, unstimulated 10T1/2 (table 5):

$$\frac{\text{total cpm in } 1\text{x}10^4 \text{ 10T1/2 cells + bFGF}}{\text{total cpm in } 1\text{x}10^4 \text{ 10T1/2 cells - bFGF}}$$

In the experiments presented here, bFGF-stimulated 10T1/2 cells, compared to the unstimulated 10T1/2, demonstrated more cell doublings, and increased ^{125}I-IUdR uptake. Table 4 illustrates that bFGF-stimulated 10T1/2 cells experienced 1 to 2 cell doublings, while the non-stimulated cells exhibited no cell doublings. Table 5 shows that there was 2 to 8 times more ^{125}I-IUdR label incorporated into bFGF-treated 10T1/2, than incorporated into quiescent 10T1/2. These observations are consistent with the idea that 10T1/2 cells incubated with bFGF, were synthesizing DNA, growing, and dividing at a faster rate than quiescent 10T1/2 incubated in DM without bFGF.

3. Lack of Membrane Damage from Granule- and Cytolysin-Treated 10T1/2 Cells

YAC-1 cells undergo rapid and extensive membrane damage from NK cells and their granules (59,99). Membrane damage may occur as a consequence of cytolysin-induced pore formation in the target cell membrane and can be quantitated by release of ^{51}Cr-labelled macromolecules from the labelled targets cell (13). Khalil has demonstrated that intact NK cells cause augmented membrane

TABLE 4: bFGF Stimulation of Quiescent 10T1/2 Increases the Number of Cell Doublings.

Experiment	bFGF (25 ng/ml)	Number of cell doublings
1	+	1.6
	-	0
2	+	1.6
	-	0.3
3	+	1.5
	-	0.3
4	+	1.2
	-	0
5	+	1.0
	-	0
6	+	1.0
	-	0.4
Mean doublings $\pm$ SE	+	1.3 $\pm$ 0.6
	-	0.2 $\pm$ 0.4

For 24 hours, $2x10^5$ cells were incubated in DM; then for 48 hours, incubated in either DM or DM with 25 ng bFGF/ml medium. The number of cell doublings for each experiment was calculated (see Results) and the mean for all 6 experiments was calculated and presented $\pm$ SE.

TABLE 5: bFGF Stimulation of Quiescent 10T1/2 Increases the Cells' Uptake of ^{125}I-IUdR.

Expt.	bFGF (25 ng/ml)	^{125}I-IUdR (cpm) incorporated per 10^4 cells	Ratio of $\frac{\text{cpm bFGF}}{\text{cpm quiescent}} \pm$ SE
1	+	11,035	2.5 ± 0.3
	-	4,420	
2	+	1,698	3.0 ± 0.1
	-	572	
3	+	8,675	7.7 ± 0.7
	-	1,127	
4	+	21,735	6.1 ± 0.2
	-	3,576	
5	+	7,900	3.7 ± 0.2
	-	2,125	

For 24 hours, $2x10^5$ cells were incubated with DM; then for 48 hours, incubated in either DM, or DM with 25 ng bFGF/ml medium. Cells were harvested and labelled with ^{125}I-IUdR (Methods) and cpm/10^4 cells was measured. ^{125}I-IUdR incorporated is expressed as the mean of triplicate values and then expressed as the ratio: cpm in bFGF-stimulated 10T1/2 to cpm in quiescent 10T1/2 ± SE.

damage in bFGF-stimulated 10T1/2 fibroblasts, but only in an 18 hour assay (72). To determine whether granule-derived cytotoxins are responsible for the bFGF-augmented cytotoxicity, ^{51}Cr-labelled 10T1/2 fibroblasts were incubated with solubilized rat NK cell granules, as described in Methods. 10T1/2 cells incubated for 4 hours with 0 to 6.57 µg/ml granule protein did not exhibit substantial ^{51}Cr release. Even at granule doses as high as 3.29 µg/ml and 6.57 µg/ml, specific ^{51}Cr release was variable and generally below 15% (see table 6). Table 6 demonstrates that isolated granules induced rapid, strong SRBC hemolysis, indicating the presence of potent cytolysin activity in the granule preparation. Additionally, ^{51}Cr release was minimal whether 10T1/2 cells were incubated with granules for 1, or for 4 hours (table 7). These observations agree with previous experiments performed by Khalil, which demonstrated that granules also failed to produce substantial ^{51}Cr release from 10T1/2 in a longer, 18 hour assay (72).

In order to determine whether bFGF-stimulated proliferation augments granule-mediated membrane damage, 10T1/2 grown in the presence or absence of bFGF, were labelled with ^{51}Cr and then exposed to RNK cell granule preparation. While bFGF increased membrane damage from 10T1/2 incubated with intact NK cells, the growth factor

TABLE 6: Granules Fail to Produce Significant Membrane Damage in bFGF-Stimulated 10T1/2

Granule concentration (µg/ml)	Percent specific ^{51}Cr release in 10T1/2 ± SE	Percent hemolysis
6.57	10 ± 7	87
3.29	12 ± 10	85
1.64	0 ± 7	77
0.82	5 ± 6	86
0.41	0 ± 0	86
0.20	0 ± 0.1	84
0.10	4 ± 4	77

^{51}Cr-labelled, bFGF-stimulated 10T1/2 were incubated with granules for 4 hours. Membrane damage was calculated as percent ^{51}Cr release and the results represent the mean of triplicate values ± SE. Spontaneous ^{51}Cr release was 25%. SRBC hemolysis was used as a positive control for granule activity.

TABLE 7: Effect of Incubation Time on the Granule-Mediated Membrane Damage of bFGF-Stimulated 10T1/2

Granule concentration (µg/ml)	Percent specific ^{51}Cr release ± SE	
	1 hour	4 hours
6.57	10 ± 6	12 ± 5
3.29	10 ± 2	11 ± 2
1.64	5 ± 4	8 ± 2
0.82	1 ± 3	5 ± 3

^{51}Cr-labelled, bFGF-stimulated 10T1/2 were incubated with granules for 1 or 4 hours. Membrane damage was calculated as percent ^{51}Cr release and the results are expressed as the mean of triplicate values ± SE. Spontaneous ^{51}Cr release was 26% in the 1 hour assay and 23% in the 4 hour assay.

failed to influence 10T1/2 membrane damage by NK granule cytotoxins. Table 8 shows that bFGF-stimulated cellular proliferation failed to enhance granule-mediated ^{51}Cr release from 10T1/2 target cells. In fact, neither bFGF-stimulated nor unstimulated, quiescent cell populations exhibited significant membrane damage: ^{51}Cr release did not exceed 13%.

Since membrane damage is believed to be initiated by pore formation in the targets' membrane, and since granule-associated cytolysin lyses cells by pore formation (144,170), we evaluated cytolysin's ability to cause ^{51}Cr release from 10T1/2 cells. Similar to ^{51}Cr release assays with granule preparations, ^{51}Cr-labelled 10T1/2 cell monolayers were incubated for 4 hours with heparin-agarose purified cytolysin. Table 9 illustrates that ^{51}Cr release was not evident from 10T1/2 cells exposed to cytolysin, even though cytolysin induced rapid and strong SRBC lysis. Cytolysin at concentrations of 2.5 to 40 lytic units/ml (where 1 lytic unit/ml equals the final cytolysin dilution producing 50% SRBC hemolysis) was capable of inducing more than 70% hemolysis. The same quantity of cytolysin produced, from 10T1/2, ^{51}Cr release below 10% (table 9). Finally, table 10 indicates that granule preparation, with or without cytolysin activity, failed to produce ^{51}Cr release.

TABLE 8: bFGF-Stimulation of Quiescent 10T1/2 Does Not Increase Granule-Mediated Membrane Damage

Granule concentration (µg/ml)	Percent specific ^{51}Cr release ± SE	
	untreated cells	bFGF (25 ng/ml)
6.57	11 ± 6	11 ± 2
3.29	9 ± 3	13 ± 4
1.64	3 ± 2	7 ± 6
0.82	4 ± 2	4 ± 6
0.41	0 ± 0	1 ± 1
0.20	0 ± 1	0 ± 2

^{51}Cr-labelled 10T1/2 (quiescent or bFGF-treated) were incubated with granules for 4 hours. Membrane damage was calculated as percent ^{51}Cr release and the results represent the mean of triplicate values ± SE. Spontaneous ^{51}Cr release was 16% for the untreated 10T1/2, and 20% for the bFGF-treated 10T1/2.

TABLE 9: Cytolysin Fails to Produce Membrane Damage in bFGF-Stimulated 10T1/2

Cytolysin concentration (LU/ml)[a]	Percent specific ^{51}Cr release from 10T1/2 ± SE	Percent hemolysis
40	6 ± 3	80
20	5 ± 5	78
10	9 ± 10	77
5	5 ± 6	76
2.5	0 ± 0	78
1.2	0 ± 0.1	63
0.6	5 ± 4	38

^{51}Cr-labelled, bFGF-stimulated 10T1/2 were incubated with pure cytolysin for 4 hours. Membrane damage was calculated as percent ^{51}Cr release and the results represent the mean of triplicate values ± SE. Spontaneous ^{51}Cr release was 25%. SRBC hemolysis was used as a positive control for cytolysin activity.

[a]Cytolysin is reported in Lytic Units/ml, where one Lytic Unit/ml = final dilution of cytolysin producing 50% SRBC hemolysis.

TABLE 10: Effect of Ca^{2+}-Pretreated Granules on Membrane Damage in bFGF-Stimulated 10T1/2

$CaCl_2$ pretreatment (mM)	Percent specific ^{51}Cr release from 10T1/2 ± SE	Percent hemolysis
0.8	3 ± 2	1
0.4	0 ± 0	0
0.2	0 ± 0	0
0.1	2 ± 4	70
0.05	0 ± 0.2	76
0.02	4 ± 6	75
0	5 ± 1	74
control[a]	13 ± 4	85

^{51}Cr-labelled 10T1/2 were incubated for 4 hours with 3.29 µg/ml of granules that were pretreated with $CaCl_2$ for 1 hour at 37° C. Membrane damage was calculated as percent ^{51}Cr release and the results are expressed as the mean of triplicate values ± SE. SRBC hemolysis was used as a positive control for granule activity.

[a]Control represents 10T1/2 cells, or SRBCs incubated with granule material which has not been pretreated with $CaCl_2$.

4. bFGF Augmented Granule-Induced Loss of Adhesion from 10T1/2 Cells

Since granules failed to induce ^{51}Cr release from 10T1/2 cells and since these cells were susceptible to NK cell cytolysis, we suspected that another type of cytotoxicity was responsible for the augmented killing induced by bFGF. One approach we used was the cytotoxicity adherence assay, which measures loss of the target cells' adhesion from their substratum. Anchorage-dependent target cells, such as L929 fibroblasts may lose adherence from their substrate upon interaction with cloned murine CTLs (1,133) or with the cytotoxin TNFα (95,132). The TNFα cytotoxic adherence assay described by Ruff and Gifford (132) and Matthews and Neale (94) was modified and employed to determine whether NK cell-derived cytotoxic granules cause 10T1/2 fibroblasts to lose adherence to their substrate, whether this effect is cytolytic for 10T1/2 targets and whether bFGF-stimulated proliferation in 10T1/2 enhances this type of cell damage.

As described in Methods, 10T1/2 fibroblasts grown as adherent monolayers were incubated for 18 to 24 hours with 0 to 6.57 µg/ml solubilized granule protein. At the end of the incubation period, loss of adherence was indirectly measured by staining the remaining adherent cells with crystal violet and calculating the cells lost

from the monolayer (see Methods). Figure 1 shows that in an 18 hour cytotoxicity adhesion assay, the granule preparation induced a dose dependent loss of adherence of 10T1/2 cells from their monolayer. The rate of 10T1/2 detachment steadily increased with granule protein concentrations of 0 to 2.0 µg/ml, and with higher concentrations, 3.0 to 6.57 µg/ml, loss of adherence appeared to plateau.

We previously observed that, while bFGF-deprived 10T1/2 cells exhibited virtually no loss of adhesion after incubation with granule proteins, bFGF-stimulated 10T1/2 experienced substantial granule-mediated loss of adhesion, with rates up to 80-100% (72). This report provides similar findings. 10T1/2 cells made quiescent by growing in serum-free, bFGF-free defined medium experienced low to moderate substrate detachment when incubated with granules. However, re-initiating proliferation in 10T1/2, by addition of bFGF to the defined medium, increased the rate of 10T1/2 cell detachment. Figure 1 shows that with 1.6 to 3.29 µg/ml of granule protein, the bFGF-stimulated 10T1/2 experienced 2 fold greater substrate detachment than did quiescent 10T1/2. At granule protein concentrations of 0.10 to 1.0 µg/ml, bFGF-stimulated 10T1/2 cells were 5 to 10 times more susceptible to loss of adhesion than the quiescent cells were. Finally, at extremely low doses of

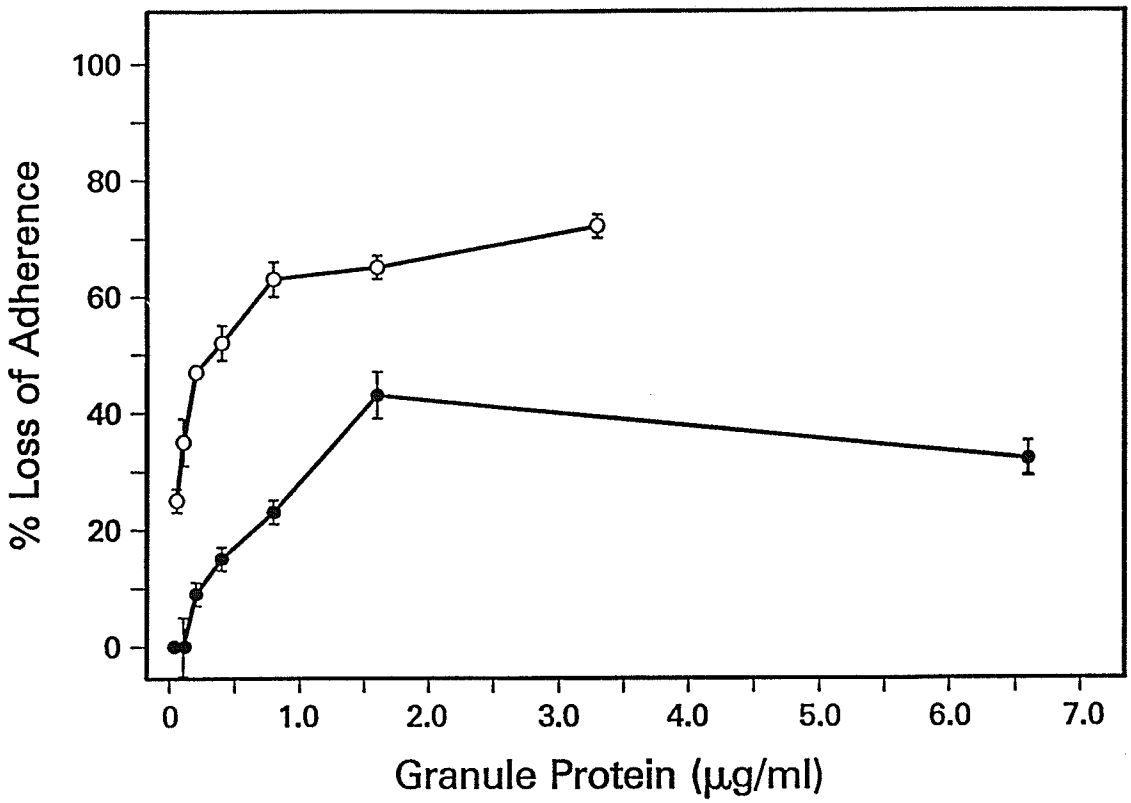

FIGURE 1: bFGF Stimulation of Quiescent 10T1/2 Augments Granule-Mediated Loss of Adhesion. Monolayers of 10T1/2 cells, incubated with or without bFGF, were exposed to solubilized granules. After 18 hours incubation at 37° C, cytotoxicity was calculated as percent loss of adhesion. The results are expressed as the mean of quadruplicate values ± SE. (○) represents bFGF-stimulated 10T1/2 cells; (●) represents unstimulated, quiescent 10T1/2 cells.

granule preparation (0.05 to 0.10 µg/ml), the quiescent 10T1/2 demonstrated no response to granules but there was 25-36% loss of adhesion in the bFGF-stimulated cells. The results indicate that 10T1/2 are susceptible to a form of cellular damage that causes the cells to lose their substrate adhesion properties in response to NK-derived granules, and this damage is augmented by bFGF-induced proliferation in 10T1/2.

While maximum loss of adherence required at least 18 hours exposure to granules, this response was evident during shorter 4 hour assays. 10T1/2 cells incubated with 3.29 µg/ml granule protein for 4 hours experienced 22% loss of adherence, while during 18 hour assays the same quantity of granule protein induced greater substrate detachment: 72% (see table 11). Detecting some damage with a shorter, 4 hour assay indicates that loss of adherence begins early in the lytic process.

Abrams and Russell have determined that CTL-mediated loss of adhesion occurred before target cell death and, using ^{51}Cr release assays, cytolysis began 1 to 2 hours after cells detached from their monolayer (1,133). With trypan blue exclusion assays, cell death was found to occur simultaneously with TNFα-induced cell detachment (95). In order to determine whether granule-mediated loss of adherence occurred before or simultaneously with 10T1/2

death, the viability of the detached cells was evaluated after incubation with granules. Adherent monolayers of 10T1/2 cells were incubated with granules for 4 or 18 hours, after which only non-adherent cells were harvested and stained with trypan blue, a dye that is excluded from living cells.

Table 11 illustrates the results of trypan blue exclusion from non-adherent 10T1/2 cells after 18 hours incubation with 3.29 µg/ml granule protein. Of the 10T1/2 cells disrupted from their monolayer, more than 86% were dead. Table 11 also shows that 10T1/2 cells incubated with 3.29 µg/ml of granules for 4 hours exhibited 22% loss of adherence. Out of these non-adherent cells, 84% were dead, as determined by trypan blue uptake. These observations suggest that granule-induced loss of adhesion is cytotoxic for 10T1/2 cells. Furthermore, since most of the non-adherent cells were dead at the end of the incubation period, even after a shorter 4 hour assay, these cells were most likely dead at the time they lost adherence from their substrate, or shortly after. Since granule-induced loss of adhesion was cytotoxic for the target cells after 4 hours incubation with granules, 10T1/2 death may begin early in the NK cytolytic process. However, it still remains unclear whether 10T1/2 loss of adhesion is a general consequence or cause of cell death.

TABLE 11: Effect of Granules on 10T1/2 Loss of Adhesion, and Viability of the Granule-Induced Detached 10T1/2

Incubation time	4 hours	18 hours
Percent loss of adhesion ± SE	22% ± 10	72% ± 2
Number of cells detached from assay plate (x 10^4)	23	83
Number of cells stained with trypan blue (x 10^4)	20	72
Percentage of the non-adherent cells that are lysed	84%	87%

Monolayers of bFGF-stimulated 10T1/2 were incubated with 3.29 µg/ml of granules for 4 or 18 hours. Loss of adhesion was calculated as described in Methods. To assess the viability of the non-adherent cells, supernatants were harvested and centrifuged. Sedimented cells were stained with trypan blue. Percent lysed cells was calculated as:

$$100 \times \frac{\text{Number of detached cells stained with trypan blue}}{\text{Total number of detached cells}}$$

5. bFGF Augmented Granule-Induced DNA Fragmentation from 10T1/2 Cells

The experiments described in the previous section indicate that NK cell cytolytic granules disrupt 10T1/2 adherent monolayers, analogous to loss of adhesion of L929 fibroblasts produced by CTLs (1,133) and TNFα (95,132). Additionally, CTLs and TNFα induce DNA fragmentation in selected target cells (95,116,166,175). Recent data show NK cells and NK cell-derived granules produce DNA fragmentation in susceptible targets (29,37,118,146). We questioned whether NK cell granules could cause DNA fragmentation in 10T1/2 fibroblasts and whether this type of damage is augmented by bFGF-stimulated proliferation. To evaluate these questions, 10T1/2 cells were labelled with ^{125}I-IUdR, cultured as adherent monolayers and exposed to NK granule preparation (0 to 6.57 µg/ml) for 2, 4, or 18 hours.

Growing and dividing cells incorporate ^{125}I-IUdR label into their DNA and the rate of ^{125}I-IUdR taken up by the cells reflects the rate of cellular proliferation and DNA synthesis (17). Release of ^{125}I-IUdR label into the assay supernatant correlates with cytolysis of the target cell and is a measure of DNA damage (36,146). Shi has found that YAC-1 cells incubated with NK granules or fragmentin plus cytolysin released ^{125}I-IUdR into the assay

supernatant (146). The DNA extracted from the ^{125}I-IUdR-labelled, cytotoxin-treated YAC-1 cells was subjected to agarose gel electrophoresis. The results show that the DNA was cleaved into oligonucleosome sized fragments. Furthermore, the DNA was degraded into small and soluble fragments, and such soluble fragments may be found in the assay supernatant. These observations show that the ^{125}I-IUdR released from labelled target cells indeed corresponds to DNA fragmentation.

In the experiments reported here, the ^{125}I-IUdR released from granule-treated 10T1/2 may represent DNA fragmentation from these targets. Figure 2 illustrates that 10T1/2 cells experienced ^{125}I-IUdR release and this isotope release increased as the granule concentration increased from 0 to 6.57 µg/ml. Table 12 indicates there was not substantially greater ^{125}I-IUdR release after 18 hours incubation with granules than after 4 hours incubation. It appears that maximum ^{125}I-IUdR release occurred after only 4 hours exposure to granules. As well, ^{125}I-IUdR release was evident very early in the 10T1/2 cytolytic process. Table 13 illustrates that ^{125}I-IUdR release could be detected as early as after only 2 hours incubation with granules.

We next compared granule-mediated ^{125}I-IUdR release from bFGF-stimulated and unstimulated 10T1/2 cells. 10T1/2

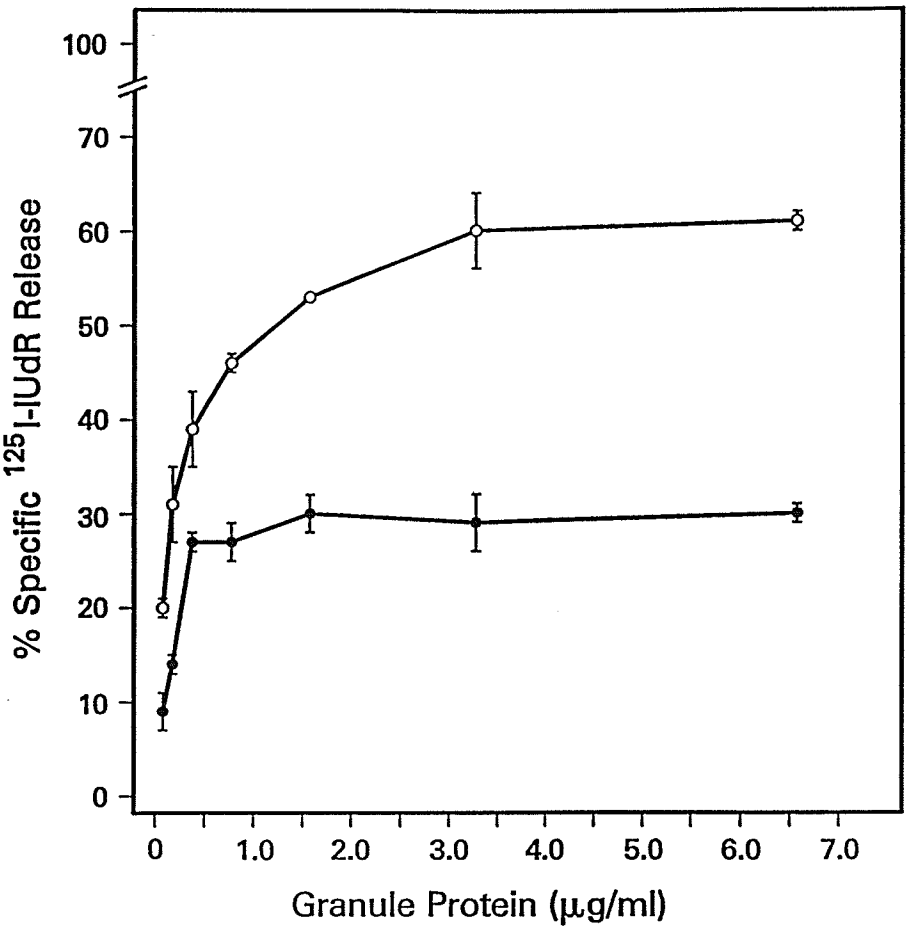

FIGURE 2: bFGF Stimulation of Quiescent 10T1/2 Augments Granule-Mediated DNA Fragmentation. Monolayers of ^{125}I-IUdR-labelled 10T1/2 were incubated with solubilized granules for 4 hours at 37° C. DNA fragmentation was calculated as percent ^{125}I-IUdR release and the results are expressed as the mean of triplicate values ± SE. Spontaneous ^{125}I-IUdR release was 4% for bFGF-stimulated and unstimulated 10T1/2. (○) represents bFGF-stimulated 10T1/2; (●) represents unstimulated, quiescent 10T1/2.

TABLE 12: Effect of Incubation Time on Granule-Mediated DNA Fragmentation in bFGF-Stimulated 10T1/2

Granule concentration (µg/ml)	Percent specific ^{125}I-IUdR release ± SE	
	4 hours	18 hours
5.54	35 ± 2	34 ± 2
2.77	32 ± 2	36 ± 1
1.38	32 ± 1	40 ± 7
0.69	27 ± 9	37 ± 8
0.35	13 ± 2	45 ± 18
0.17	13 ± 10	37 ± 14
0.09	5 ± 4	27 ± 8

^{125}I-IUdR-labelled 10T1/2 were incubated with granules for 4 or 18 hours. DNA fragmentation was calculated as percent ^{125}I-IUdR release and the results are expressed as the mean of triplicate values ± SE. Spontaneous ^{125}I-IUdR release was 5% for the 4 hour assay and 12% the 18 hour assay.

TABLE 13: Granules Induce DNA Fragmentation in bFGF-Stimulated 10T1/2 in a 2 Hour Assay

Granule concentration (µg/ml)	Percent specific ^{125}I-IUdR release from 10T1/2 ± SE	Percent hemolysis
5.54	27 ± 6	99
2.77	25 ± 3	97
1.38	19 ± 4	94
0.69	11 ± 3	93
0.35	6 ± 2	85
0.17	7 ± 5	72
0.09	2 ± 0.5	51

^{125}I-IUdR-labelled, bFGF-stimulated 10T1/2 cells were incubated with granules for 2 hours. DNA fragmentation was calculated as the percent ^{125}I-IUdR release and the results are expressed as the mean of quadruplicate values ± SE. Spontaneous ^{125}I-IUdR release was 4%. SRBC hemolysis was used as a positive control for granule activity.

were made quiescent in serum-free, bFGF-free defined medium and proliferation was re-initiated by addition of bFGF to the medium. With the methods described previously, these cells were labelled with ^{125}I-IUdR, plated as adherent monolayers and then incubated with 0 to 6.57 µg/ml of granule protein for 4 hours. Figure 2 illustrates that bFGF-stimulated proliferation in 10T1/2 augmented granule-mediated ^{125}I-IUdR release compared to the quiescent cells. The granules, at all concentrations tested, produced 2 fold greater ^{125}I-IUdR release from bFGF-stimulated cells than from the quiescent 10T1/2 (figure 2). Also, quiescent 10T1/2 cells achieved maximum ^{125}I-IUdR release at very low granule concentrations: that is, granule concentrations greater than 0.4 µg/ml failed to cause significantly more isotope release than the lower concentrations produced.

6. The Effects of Heparin-Agarose Purified Cytolysin on Loss of Adhesion and DNA Fragmentation from 10T1/2 Cells

We previously reported that cytolysin could not induce extensive membrane damage in 10T1/2 cells, regardless of whether the cells were bFGF-stimulated or not. We also reported that NK cell-derived granules produced both loss of adhesion and DNA fragmentation in 10T1/2 targets, and both forms of damage were augmented by bFGF-stimulation of the target cells. Our question was whether cytolysin

participated either directly or indirectly in the granule-mediated loss of adherence and/or DNA fragmentation, and whether cytolysin mediated this bFGF-augmented cytolysis of 10T1/2. To evaluate this question, monolayers of 10T1/2 cells were treated with heparin-agarose purified cytolysin in cytotoxic adhesion assays and ^{125}I-IUdR release assays.

Purified cytolysin produced neither loss of adhesion (see figure 3) nor ^{125}I-IUdR release (see figure 4) from 10T1/2 targets. Figure 3 demonstrates that purified cytolysin produced substantial SRBC hemolysis, reaching 70-90% hemolysis at 1.3 to 2.6 lytic units/ml cytolysin (where 1 lytic unit/ml equals the final cytolysin dilution producing 50% hemolysis). Cytolysin at 2.6 lytic units/ml produced only 9% loss of adherence, and concentrations below this produced no loss of adhesion. In figure 4, cytolysin similarly caused substantial SRBC hemolysis but failed to produce ^{125}I-IUdR release from 10T1/2 cells. From 1.2 to 39 lytic units/ml of cytolysin caused 60%-90% hemolysis but similar cytolysin concentrations invoked absolutely no ^{125}I-IUdR release from 10T1/2 cells. Figure 4 also shows that 0.41 to 6.57 µg/ml of granules produced about 45% ^{125}I-IUdR release from 10T1/2 cells. These data indicate that, unlike granules, cytolysin cannot disrupt the adherence of 10T1/2 cells from their substrate nor fragment 10T1/2 cells' DNA.

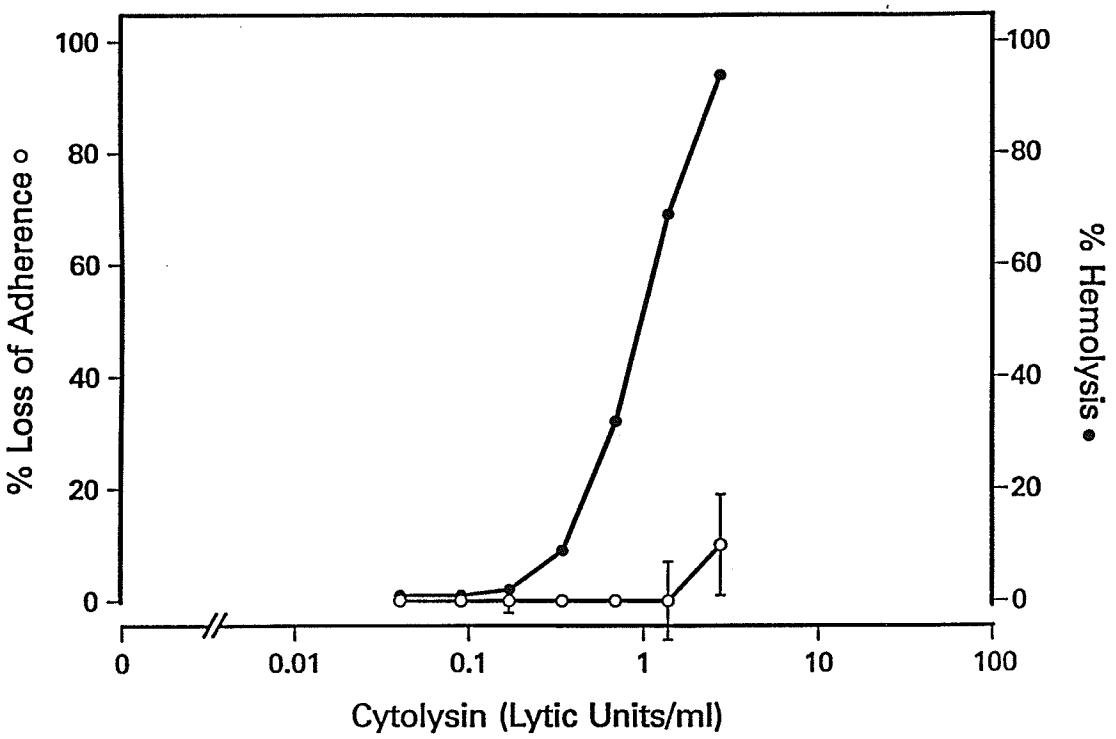

FIGURE 3: Cytolysin Fails to Induce Loss of Adhesion in bFGF-Stimulated 10T1/2. Monolayers of bFGF-stimulated 10T1/2 cells were incubated with heparin-agarose purified cytolysin for 18 hours at 37° C. Cytotoxicity was calculated as percent loss of adhesion, and results are expressed as the mean of triplicate values ± SE. SRBC hemolysis was used as a positive control for cytolysin activity. (O) represents 10T1/2 cells; (●) represents SRBCs. One lytic unit = final dilution cytolysin producing 50% SRBC hemolysis.

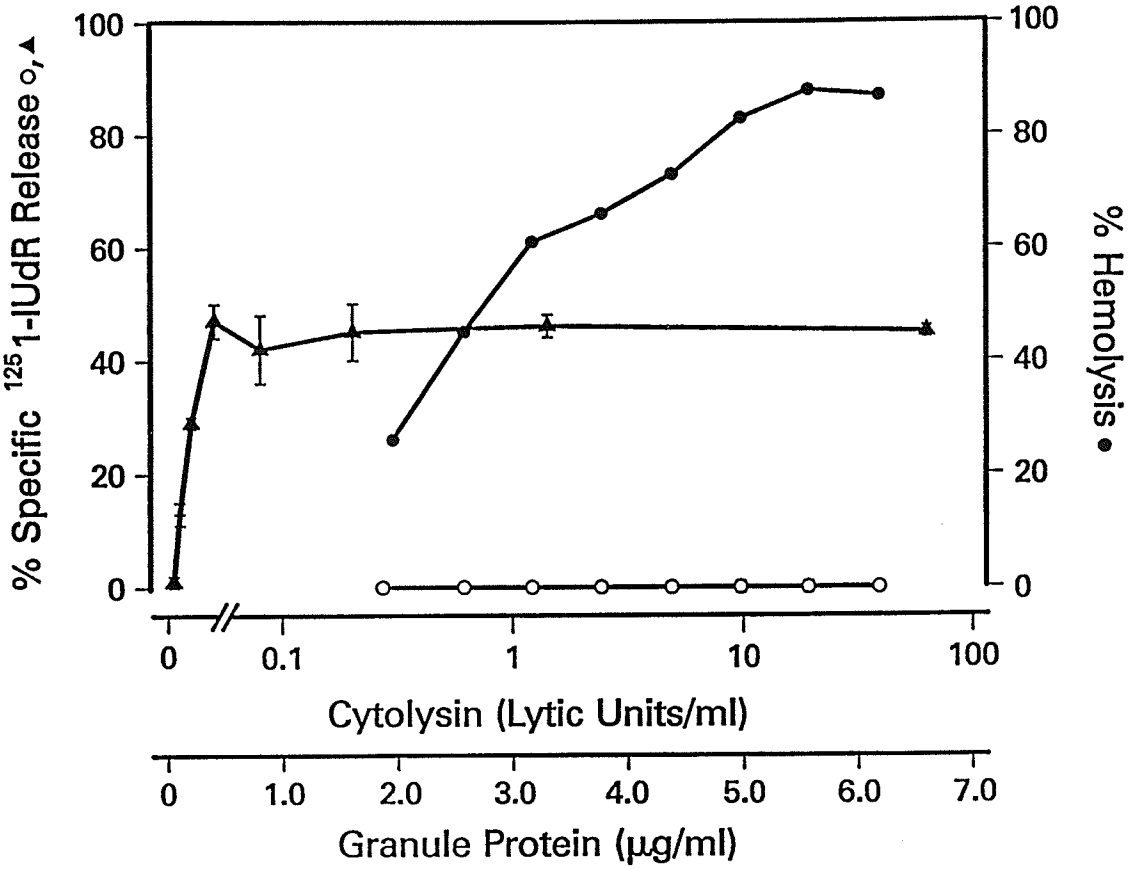

FIGURE 4: Cytolysin Fails to Induce DNA Fragmentation in bFGF-Stimulated 10T1/2. Monolayers of ^{125}I-IUdR-labelled 10T1/2 were incubated with granules or cytolysin. DNA fragmentation was measured as percent ^{125}I-IUdR release; the results are expressed as the mean of triplicate values ± SE. Spontaneous ^{125}I-IUdR release was 8% for granules and 7% for cytolysin. SRBC hemolysis was used as positive control for cytolysin activity. (▲) represents granules; (○) represents cytolysin; (●) represents SRBCs. One lytic unit = final dilution cytolysin producing 50% hemolysis.

The experiments described in figures 3 and 4 also demonstrate that stimulating 10T1/2 cells with bFGF did not enhance cytolysin-mediated loss of adherence or ^{125}I-IUdR release. 10T1/2 cells actively proliferating under the influence of bFGF remained completely resistant to loss of adhesion and DNA fragmentation when incubated with purified cytolysin.

7. The Effects of Cytolysin-Depleted Granules on Loss of Adhesion and DNA Fragmentation in 10T1/2 Cells

While cytolysin itself could not damage 10T1/2 cells in the same manner as whole granule preparations, the possibility remained that cytolysin participates, as a secondary effector molecule, in granule-mediated loss of adhesion and DNA fragmentation. If cytolysin assists another granule effector molecule in the damage of 10T1/2 cells, then depleting its activity from the granule preparations should diminish or even abolish granule-mediated loss of adherence and/or ^{125}I-IUdR release. In order to look at this effect, adherent monolayers of 10T1/2 cells were incubated with 3.29 µg/ml of granule preparation that was depleted of cytolysin activity. Cytolysin was removed from the granules by pretreating them with millimolar concentrations of $CaCl_2$ for one hour at 37^{o} C, or by chelating extracellular Ca^{2+} with EGTA.

Tables 14 and 15 demonstrate that cytolysin in the granule preparation was inactive at 1.6 to 0.2 mM $CaCl_2$ and was active at 0.1 to 0 mM $CaCl_2$. Granule preparations devoid of cytolysin activity still produced substantial loss of adhesion and ^{125}I-IUdR release from 10T1/2. Table 14 shows that 10T1/2 cells still experienced loss of substrate adherence with cytolysin-depleted granule material. The rate of 10T1/2 detachment mediated by granules containing active cytolysin (42-61%) was equivalent to that produced by granules lacking cytolysin activity (51-65%). Similar results are seen in table 15 for ^{125}I-IUdR release. Depletion of cytolysin from granule preparations did not abolish ^{125}I-IUdR release from 10T1/2 cells. Furthermore, the quantity of ^{125}I-IUdR released from 10T1/2 cells exposed to granules containing active cytolysin (44-50%) was similar to that from 10T1/2 exposed to granules lacking cytolysin activity (43-53%). These observations indicate that removal of cytolysin did not inhibit either granule-associated loss of adherence or DNA fragmentation in 10T1/2 cells. Furthermore, depleting cytolysin did not even diminish the rate of target cell detachment and DNA damage, suggesting that cytolysin may not be involved with these mechanisms of cellular damage.

Cytolysin's action against target cells requires Ca^{2+} in the assay medium. Removal of extracellular Ca^{2+} with

TABLE 14: Effect of Ca^{2+}-Pretreated Granules on the Adhesion Properties of bFGF-Stimulated 10T1/2

$CaCl_2$ pretreatment (mM)	Percent loss of adhesion of 10T1/2 ± SE	Percent hemolysis
1.6	51 ± 3	0
0.8	59 ± 3	0
0.4	57 ± 3	1
0.2	65 ± 4	2
0.1	61 ± 6	69
0.05	53 ± 4	97
0.02	60 ± 8	95
0	42 ± 6	96
control[a]	49 ± 3	98

bFGF-stimulated 10T1/2 monolayers were incubated for 18 hours with granules (3.29 µg/ml) that were pretreated with $CaCl_2$ for 1 hour at 37° C. Cytotoxicity was calculated as percent detached cells and the results represent the mean of triplicate values ± SE. SRBC hemolysis was used as a positive control for granule activity.

[a]Control represents 10T1/2 cells, or SRBCs incubated with granules which have not been pretreated with $CaCl_2$.

TABLE 15: Effect of Ca^{2+}-Pretreated Granules on DNA Fragmentation in bFGF-Stimulated 10T1/2

$CaCl_2$ pretreatment (mM)	Percent specific ^{125}I-IUdR release from 10T1/2 ± SE	Percent hemolysis
0.8	43 ± 6	0
0.4	53 ± 13	0
0.2	47 ± 3	0
0.1	50 ± 4	13
0.05	46 ± 7	39
0	44 ± 11	41
control[a]	47 ± 3	38

bFGF-stimulated, ^{125}I-IUdR-labelled 10T1/2 were incubated for 4 hours with 3.29 µg/ml of granules that were pre-treated with $CaCl_2$ for 1 hour at 37° C. DNA fragmentation was calculated as percent ^{125}I-IUdR release and the results are expressed as the mean of triplicate values ± SE. SRBC hemolysis was used as a positive control for granule activity.

[a]Control represents 10T1/2 cells, or SRBCs incubated with granule material which has not been pretreated with $CaCl_2$.

the chelating agent EGTA destroys cytolysin's cytotoxic activity (166). For this report, 10T1/2 monolayers were incubated in medium containing 1.1 mM EGTA, a concentration that exceeds the extracellular Ca^{2+} concentration of the defined medium. Increasing concentrations of $CaCl_2$ were added back into the assay wells, and then the cells were incubated with 3.29 µg/ml of granules for 4 hours. Table 16 illustrates that in the assay wells containing excess EGTA (0.7 - 1.1 mM $CaCl_2$), cytolysin's hemolytic action against SRBCs was blocked. Adding $CaCl_2$ to the assay medium in excess of the EGTA content (1.3 - 1.7 mM $CaCl_2$) restored cytolysin activity against SRBCs. ^{125}I-IUdR release from 10T1/2 cells was evident when extracellular Ca^{2+} and cytolysin activity were absent, as well as when extracellular Ca^{2+} and cytolysin activity were present (table 16), showing that this damage of 10T1/2 occurred without any benefit of cytolysin activity.

TABLE 16: Effect of Extracellular Ca^{2+} on Granule-Mediated DNA Fragmentation in bFGF-Stimulated 10T1/2

$CaCl_2$ (mM)	EGTA (mM)	Percent specific ^{125}I-IUdR release from 10T1/2 ± SE	Percent hemolysis
0.7	1.1	21 ± 4	1
0.9	1.1	29 ± 5	0
1.1	1.1	34 ± 8	0
1.3	1.1	27 ± 4	3
1.5	1.1	25 ± 3	38
1.7	1.1	28 ± 7	72

bFGF-stimulated, ^{125}I-IUdR-labelled 10T1/2 were incubated with 3.29 µg/ml of granules containing 1.1 mM EGTA. From 0.7 to 1.7 mM $CaCl_2$ was added to the cells and incubated for 4 hours. DNA fragmentation was calculated as percent ^{125}I-IUdR release and the results represent the mean of triplicate values ± SE. Spontaneous DNA fragmentation was below 17%. SRBC hemolysis was used as a positive control for granule activity.

CHAPTER 6: DISCUSSION

Many previous investigations have demonstrated that NK cells lyse a wide variety of target cells, including tumor cells, virally-infected cells, poorly differentiated cells, and some normal cells such as fibroblasts (107,153). NK-mediated cytolysis proceeds through three discrete steps: (1) recognition of and binding to the target cell; (2) activation of the NK cell to deliver its lethal hit; (3) cytolysis, or lethal hit of the target cell (13,166). NK cell cytoplasmic granules contain potent lytic molecules which exhibit the same cytolytic activity of the intact NK cell and participate in the lethal hit (59,99,166). NK cell granule-associated cytotoxins include cytolysin, a complement-like molecule (59). Recently, two novel cytotoxins have been purified from rat NK cell granules. Shi et al have described a DNA fragmenting cytotoxin (146). Sayers' group purified a molecule that alters growth and adhesion properties of tumor cells (136).

The mechanism by which NK cells and their granules cause target cell lysis remains incompletely understood. In some target cells, including YAC-1 lymphoma, NK cells and NK cytotoxic granules cause extensive membrane damage (59,99,146). This damage is thought to result from the production of transmembrane pores in the cell's plasma membrane, leading to cellular ion concentration changes

which ultimately cause osmotic lysis of the affected cell. With development of pores and intracellular ion changes, the osmotic pressure gradient between the cell and its environment may change, causing excess water to flow into the cell. These effects could lead to swelling and rupture of the cell (13,166). An alternative model of cytolysis suggests that pores in the cell membrane cause Ca^{2+} to move into the cell, down its concentration gradient (75). This increase in intracellular Ca^{2+}, if large enough, may lead to necrosis of the cell by stimulating derangements in cellular functions (126,175). Increases in internal Ca^{2+} may: activate cellular proteases, phospholipases, endonucleases; inactivate cellular ionic pumps; disrupt cellular organelles and the cytoskeleton (75,126,168,169). However, regardless of whether these ion-dependent cellular derangements lead to osmotic lysis or necrosis, both are passive forms of cell death and both may lead to extensive fragmentation of the plasma membrane (175).

Extensive membrane disruption can be measured with the ^{51}Cr release assay (17,144). ^{51}Cr binds to large macromolecules within the target cell, and the isotope is released into the supernatant upon extensive fragmentation of the cell's plasma membrane.

Cytolysin is a 60 kD protein isolated from NK cell lytic granules and has the same pore-forming capability

as complement and CTL-derived perforin (170). Cytolysin appears to be at least partially responsible for the membrane damage to YAC-1 cells, although it remains unclear whether this damage is the result of osmotic lysis or necrosis (13,59,170,175). YAC-1 cells exposed to NK cells, NK granules or pure cytolysin exhibit maximum ^{51}Cr release within one hour (59). The membrane damage is very rapid, and substantial YAC-1 lysis occurs early in the cytolytic process. This is consistent with the idea that extensive membrane damage, caused by the formation of transmembrane pores, may be a primary cause of death in YAC-1 cells. Although cytolysin has been shown to be cytotoxic against YAC-1 cells, cytolysin-mediated damage may not be the NK cell's only mechanism of lysis. Some target cells, such as 10T1/2 fibroblasts, are susceptible to NK-mediated lysis but are not damaged by cytolysin. Furthermore, while NK cells produce DNA fragmentation in certain target cells, cytolysin cannot induce DNA fragmentation (4,38,146). These observations indicate that cytolysin may not be essential for NK-mediated cytolysis in all target cells. Furthermore, there are CTLs which lack perforin and/or cytoplasmic granules but are still capable of lysing target cells (4). However, NK cells that lack cytolysin or granules and can still lyse target cells have not been found, and antibodies directed at NK granules

block cytolysis. It appears that, although cytolysin may not be cytotoxic to all cells, granule-mediated damage is a prime mechanism in NK-mediated cytotoxicity.

Previous experiments have demonstrated that 10T1/2 fibroblasts are susceptible to cytolysis by NK cells (40,51,72). ^{51}Cr release assays using intact NK cells demonstrated membrane damage in 10T1/2 fibroblasts (72). However, 18 hour assays and intact NK cells were required to exhibit this damage. Experiments reported here show that cytoplasmic granules isolated from the RNK leukemia line killed 10T1/2 but not through ^{51}Cr release (or membrane damage). NK granules failed to induce substantial membrane damage in 1 hour and 4 hour ^{51}Cr release assays. Although NK cells induced membrane damage in 10T1/2, Khalil has found that NK cell granules did not cause substantial membrane damage in 10T1/2 during 18 hour assays (72). In Khalil's granule experiments, adherent monolayers of 10T1/2 target cells were not used, as described in this report. An 18 hour granule assay performed with adherent 10T1/2 targets may demonstrate greater ^{51}Cr release than what was seen in Khalil's experiments. It remains unclear whether the lack of membrane damage in Khalil's experiments reflects the difference in assay techniques, or reflects 10T1/2 resistance to granule-mediated membrane damage.

Since cytolysin has been found to induce rapid and

early membrane damage in YAC-1 cells, we evaluated whether cytolysin would be able to induce membrane damage, or ^{51}Cr release from 10T1/2 fibroblasts. The data illustrate that heparin-agarose purified cytolysin at concentrations capable of producing 90% SRBC hemolysis failed to cause ^{51}Cr release from 10T1/2 cells.

This lack of membrane damage in 10T1/2 is different from what is seen with YAC-1 cells. 10T1/2 fibroblasts do not undergo early, rapid and extensive ^{51}Cr release when treated with NK granules or cytolysin, and intact NK cells induce ^{51}Cr release from 10T1/2 but only after prolonged incubation. Furthermore, while an 18 hour granule assay with adherent 10T1/2 may demonstrate greater ^{51}Cr release than that reported by Khalil, such damage would still be a late event. This suggests that extensive membrane damage may not be a primary or immediate cause of 10T1/2 death. While it remains possible that granules induce some degree of membrane damage in 10T1/2 targets, this damage may be minor and not extensive enough for ^{51}Cr release to occur. Any ^{51}Cr release seen may occur as a secondary and indirect result of some other form of damage inflicted upon the target cell. While NK cells carry granule-associated cytotoxins capable of inducing extensive membrane damage in some target cells, this damage does not seem to be a primary and significant lytic event for 10T1/2 cells.

While Khalil has found that bFGF-stimulated 10T1/2 exhibited increased ^{51}Cr release when incubated with NK cells, this effect occurred during 18 hour assays and required intact NK cells (72). The experiments reported here demonstrate that stimulating proliferation in quiescent 10T1/2 fibroblasts with bFGF did not increase the cells' susceptibility to NK granule-mediated membrane damage. In 4 hour assays, bFGF-stimulated and quiescent 10T1/2 were equally resistant to granule-induced ^{51}Cr release. Extensive membrane damage does not appear to be a primary mechanism of damage in the growth factor enhanced cytolysis of 10T1/2 cells.

Since granule-mediated 10T1/2 cytolysis and bFGF-augmented NK sensitivity seem to be caused by lytic mechanisms other than extensive membrane damage, loss of target cell adhesion and DNA fragmentation were evaluated. Cytotoxic cells, such as CTLs and NK cells can induce in certain cells damage other that membrane disintegration. Such injury includes loss of the target cells' adherence from their substrate, and this can be produced by CTLs or TNFα (1,33,95,132,133). Furthermore, recent experiments have shown that an NK granule-derived serine protease is capable of producing changes in rat tumor cell adherence properties (136). In the cytotoxicity adherence assay, we measured loss of adherence in monolayers of 10T1/2

incubated with NK-derived granules. These experiments demonstrate that granules disrupted the monolayers in a dose and time dependent manner.

While maximum loss of adhesion occurred after 18 hours incubation with granule cytotoxins, this effect was also evident in less time. After 4 hours incubation with NK granules, 10T1/2 monolayers experienced loss of adhesion, although to a lesser extent than with the 18 hour assays. Although granule-induced loss of adherence does not seem to be a rapid and early event, it may begin early in the 10T1/2 cytolytic process. While maximum loss of adherence occurred with 18 hour assays, and while 10T1/2 experienced ^{51}Cr release only with 18 hour assays and intact NK cells, it remains unclear whether there is any relationship between 10T1/2 loss of adhesion and membrane damage. The rate of ^{51}Cr release in a 4 hour assay is equivalent to the rate of loss of adhesion in a 4 hour assay. Perhaps some form of membrane damage modifies 10T1/2 adhesion properties, and eventually leads to disruption of the monolayers before extensive ^{51}Cr release can be detected. However, the exact nature of the damage that causes 10T1/2, and other cells, to lose adherence from their substrate remains unknown.

As detailed earlier, viability of the non-adherent 10T1/2 was assessed with trypan blue, immediately after

incubation with granules in a cytotoxicity adhesion assay. More than 80% of the granule-disrupted, non-adherent 10T1/2 cells excluded trypan blue, indicating that most of the detached cells were dead. Furthermore, since more than 80% of the non-adherent 10T1/2 cells were dead after only 4 hours incubation with granules, we suggest 10T1/2 were likely dead at time of detachment, or shortly after.

Extensive trypan blue uptake was seen with 4 and 18 hour granule assays but similar ^{51}Cr release assays did not demonstrate significant membrane damage. It is unclear why 10T1/2 were permeable to trypan blue but not to ^{51}Cr. However, trypan blue exclusion was measured only on 10T1/2 cells already disrupted from the adherent monolayer, a more selective population of cells. The process of cell detachment and/or the effect of floating in the supernatant may alter the membrane permeability of these non-adherent cells. Additionally, 10T1/2 cell membranes might be permeable to trypan blue but may not be fragmented enough to allow significant ^{51}Cr release. Granules may induce membrane permeability changes in 10T1/2 but the membrane disruption could be minor and not great enough to allow release of ^{51}Cr from the cells.

10T1/2 cells were killed before, during or immediately after detaching from their substrate, and this damage seems to resemble loss of adherence produced by TNFα rather than

by CTLs. In CTL-mediated loss of adhesion, L929 target cells did not lyse until 1 to 2 hours after detachment, suggesting that loss of adhesion is not a consequence of cell death (1,133). On the other hand, in TNFα-mediated loss of adhesion, L929 cells were dead at the time they detached from their substrate or soon after (95), and it is unclear whether cell death is a cause or consequence of cell detachment. However, in CTL-mediated loss of adhesion, Abrams et al measured L929 cell viability using ^{51}Cr release assays (1). It is possible that the prolonged viability of CTL-treated L929 cells may reflect the use of different assay systems. Russell et al looked at cell viability of CTL-detached L929 cells with trypan blue exclusion assays (133). They found that most detached cells in medium without Ca^{2+} were alive, but when the detached cells were incubated with Ca^{2+}, there were fewer living cells. The rate of cell death in these latter non-adherent cells approached the rate of cell death for detached 10T1/2 cells.

CTLs, TNFα and NK cells can damage the DNA of susceptible target cells (29,82,91,119,175). There are conflicting views regarding the DNA fragmenting-capacity of cytolytic granules. Investigators such as Duke and Gromkowski find that CTL granules do not produce DNA fragmentation in target cells (39,52). However, Podack's

group has found that CTL granules degrade target cell DNA (119). Recently, Shi has isolated a cytotoxin (fragmentin) from NK cell granules and fragmentin produces DNA fragmentation in YAC-1 and other lymphoma target cells (146).

As described in Results, Shi has found that ^{125}I-IUdR released from YAC-1 cells into the supernatant directly corresponds to the cleavage of DNA into soluble, oligonucleosome-sized fragments (146). This report demonstrates that 10T1/2 cells released ^{125}I-IUdR into the supernatant when incubated with NK cell-derived granules. This ^{125}I-IUdR release seems to indicate that DNA fragmentation has occurred in these target cells.

DNA fragmentation in 10T1/2 was dependent on dose of and time exposed to granules. Furthermore, DNA damage in 10T1/2 appeared to occur early: maximum ^{125}I-IUdR release occurred during 4 hours incubation with granule cytotoxins, and ^{125}I-IUdR release was evident with 2 hour assays. Additionally, at the larger granule concentrations, DNA fragmentation was equivalent in 4 and 18 hour experiments. But at lower granule concentrations, there was greater ^{125}I-IUdR release from 10T1/2 during the 18 hour assays. This observation does not appear to be the result of a greater degree of error in the 18 hour assay, since this kind of variability should be cancelled out with the calculations for specific ^{125}I-IUdR release. The smaller

quantities of granules may require a longer assay in order to exert any effect on the target cells.

Release of ^{125}I-IUdR from granule-treated 10T1/2 does not seem to be merely the product of membrane damage, because it has been determined that membrane damage is not an early event in granule-mediated 10T1/2 cytolysis. Granule-mediated ^{51}Cr release from 10T1/2 cells during a 4 hour assay is not sufficient to account for the ^{125}I-IUdR released during a similar 4 hour assay. The reason for this discrepancy could be related to DNA fragment size and the extent of disrution of 10T1/2 cell membranes. When target cell DNA is cleaved, it is cleaved into small and soluble fragments (36,146). ^{51}Cr binds to larger and less soluble macromolecules within the target cells, and may require very extensive membrane fragmentation for release into the supernatant. In contrast, isotope-labelled DNA fragments may be released from cells with minor disruption of the membrane. Granules could induce membrane changes in 10T1/2 but this damage may involve only small increases in membrane permeability. This damage would allow release of the smaller, more soluble ^{125}I-IUdR-labelled DNA fragments from 10T1/2. However, such membrane disruption may not be great enough for 10T1/2 to release the large, less soluble ^{51}Cr-labelled macromolecules into the supernatant. Any early membrane damage in 10T1/2 might create only minor

changes in membrane permeability, while more extensive membrane disruption occurs secondary to rapid and early DNA fragmentation.

The relationship between loss of adhesion and DNA damage in 10T1/2 also remains to be defined. The lesion(s) causing both loss of adhesion and DNA fragmentation in 10T1/2, as well as in other target cells, is not fully known. Granule-mediated cytolysis may be caused by one lesion that leads to both DNA damage and loss of adhesion, or by two independent lesions.

Inducing proliferation in quiescent 10T1/2 cells with bFGF increased their susceptibility to cytolysis by intact NK cells (72). Basic-FGF-stimulated proliferation also rendered 10T1/2 cells more susceptible to NK granule-mediated cytolysis but not through extensive membrane damage. 10T1/2 cells made quiescent in serum-free, bFGF-free defined medium were moderately susceptible to NK granule-induced loss of adherence and DNA fragmentation. Re-initiating proliferation in quiescent 10T1/2 cells with bFGF increased this granule-associated cytolysis. With NK cell granules, bFGF-stimulated and proliferating 10T1/2 cells experienced 2 to 10 times greater loss of substratum adherence than did quiescent cells. The ^{125}I-IUdR release assays demonstrated similar findings. Compared to the quiescent 10T1/2, re-initiating cell proliferation with

bFGF at least doubled the DNA fragmentation from these stimulated 10T1/2.

It may be argued that bFGF-augmented ^{125}I-IUdR release is a result of the increased quantities of ^{125}I-IUdR incorporated into bFGF-stimulated 10T1/2 because these cells are proliferating more than quiescent cells, and the isotope is taken up more rapidly in growing and dividing cells. However, the calculations for specific DNA fragmentation effectively account for the unequal quantities of isotope taken up by variable cell populations. The different quantities of ^{125}I-IUdR released from the proliferating and quiescent 10T1/2 cell populations appear to reflect bFGF-modified susceptibility of the fibroblasts to granule cytotoxins.

Cytolysin was evaluated as the effector molecule responsible for bFGF-enhanced 10T1/2 cytolysis. Because extensive membrane damage is not a primary mechanism of 10T1/2 cytolysis (bFGF-stimulation or not) and since cytolysin generally lyses target cells through membrane damage, we thought that cytolysin would not be responsible for the observed bFGF-augmented loss of adhesion and DNA fragmentation. Russell et al have found that although CTLs induce loss of adhesion, this effect cannot be reproduced with complement plus antibodies (1,133). While Hameed et al have reported that DNA fragmentation may

occur through perforin and pore-formation (55), the vast majority of investigators have failed to associate cytolysin, perforin or complement activity with DNA fragmentation (4,38,52,119,167). Even though cytolysin-mediated pore-formation may increase target cell intracellular Ca^{2+}, which causes activation of an autolytic cell death (75), DNA fragmentation does not seem to occur. However, cytolysin is absolutely required for NK granule- and fragmentin-induced DNA degradation in YAC-1 cells (146). The cytotoxin cannot yet be disregarded in the NK granule-mediated cytolysis of 10T1/2, and the effect of cytolysin on 10T1/2 loss of adhesion and DNA fragmentation was evaluated.

Heparin-agarose purified cytolysin, at concentrations capable of inducing nearly 100% SRBC hemolysis, failed to cause loss of adhesion or DNA fragmentation in 10T1/2 (plus or minus bFGF). Alternatively, cytolysin was removed from granule preparations by preincubating granules in excess Ca^{2+} plus heat or by depleting extracellular Ca^{2+} with EGTA. These procedures abolished cytolysin activity from the granules but did not inhibit cytolysis of 10T1/2. Granules produced the same rate of loss of adherence and DNA fragmentation regardless of whether cytolysin activity was present or not. Such observations indicate that cytolysin itself does not produce loss of 10T1/2 adherence

or DNA fragmentation, and cytolysin is not responsible for bFGF-augmented lysis of these target cells. Also, unlike fragmentin-mediated DNA fragmentation in YAC-1 targets (146), cytolysin does not, through some secondary mechanism, assist another granule-associated cytotoxin to produce target cell detachment and DNA damage. The inability of cytolysin to induce bFGF-augmented loss of adherence and DNA fragmentation from 10T1/2 argues that some other granule-associated cytotoxin mediates these effects without any associated action from cytolysin.

These experiments show that NK cell granules, like CTLs and TNFα, cause anchorage-dependent 10T1/2 cells to lose adherence from their substrate. NK granule-induced loss of adhesion differs from the CTL-mediated event in several respects. Russell's group reports that CTL-mediated loss of adhesion requires intact CTLs, does not require calcium and cannot be duplicated with complement plus antibodies (1,133). CTLs which lack perforin and protease also induce loss of adhesion, suggesting that granules may not be involved in this damage. The CTL-mediated damage is rapid and early, and target cell death occurs 1 to 2 hours after cell detachment. TNFα-mediated loss of adhesion has longer kinetics with optimal effect occurring in 10-24 hour assays (95). Additionally, L929 cells are lysed at the time they lose adherence or shortly

afterwards, rather than several hours after detachment.

NK granule-induced loss of adhesion in 10T1/2 cells is produced by isolated granule preparations and does not require intact NK cells. This disruption of 10T1/2 monolayers begins early but maximum loss of adhesion requires 18 hours incubation with granules. Finally, most of the cells which have lost adherence from the monolayer seem to be dead, and probably were killed either at the time they detached from the monolayer, or shortly after.

The observations above raise the possibility that TNFα within NK granules produces this damage. Some investigators have reported that TNFα is produced and secreted by $CD16^+$ NK cells but none have detected TNFα in the cytoplasmic granules (109,116). It remains controversial whether TNFα exists in all NK cells or only in certain subsets, since other investigators have reported that lymphocytes do not produce TNFα at all (95). Khalil failed to find TNFα in rat NK cell cytoplasmic granules and observed that recombinant TNFα failed to lyse 10T1/2 cells in a cytotoxicity adhesion assay (72). Such observations indicate that classical TNFα is not likely the cytotoxin responsible for granule-mediated 10T1/2 loss of adhesion.

While classical TNFα may not mediate loss of adhesion, a TNFα-like molecule may produce this damage,

since cytotoxins that resemble TNFα have been found in cytotoxic effector cells. Liu et al have found that CTL granules contain a 50-70 kD molecule that is functionally, serologically, but not structurally related to TNFα (86). While a similar molecule has not been found in NK cell granules, it is possible that related cytotoxins exist. Natural killer cytotoxic factor (NKCF) is a TNFα-like cytotoxin, whose cytolytic activities are partially inhibited by antibodies against TNFα (18). But NKCF has not been localized within the cytoplasmic granules of NK cells (166).

Granules are also rich in proteases that may produce loss of adhesion in 10T1/2 (13,166). Sayers et al have purified a serine protease from rat NK granules; the protease is homologous to RNKP-1 (136). This serine protease causes cell rounding with the formation of large aggregates, and growth inhibition in rodent tumor cells. The association between this and other NK granule serine esterases with 10T1/2 loss of adhesion remains to be defined.

In addition to their effect on target cell adherence, TNFα and CTLs induce extensive fragmentation of target cell DNA, often through the process known as apoptosis (95,116,119,175). As opposed to pore-formation which may produce extensive membrane damage, there is evidence for

another form of target cell death which is known as the internal disintegration of the cell, or apoptosis. Apoptosis occurs in target cells, where upon activation of certain signals, the cells undergo programmed cell death (91,146,175). This form of damage is characterized by: vacuolization of the cytoplasm; membrane blebbing with loss of substrate adherence; chromatin condensation and fragmentation of DNA into oligonucleosome-sized fragments. Apoptosis is stimulated in specific target cells through their contact with glucocorticoids (31,97), CTLs (157,175), TNFα (91) and NK cells (146). It is not clear whether the DNA fragmentation produced by CTLs, TNFα and NK cells is always the result of apoptosis. Cytolysin or perforin is unable to induce this type of damage in target cells (175). The degraded DNA is separated into oligonucleosome-sized fragments indicating that an endonuclease may be responsible for the final cleavage of DNA (31,36,157).

For apoptosis and DNA fragmentation to occur within certain cells, active RNA and protein synthesis in the target cell may be required. For example, glucocorticoid-mediated apoptosis in thymocytes depends on active protein synthesis in the target cells (36,91). Zychlinsky's group has found that actinomycin D-, puromycin-, or emetine-mediated inhibition of macromolecular synthesis in P815, EL-4, and YAC-1 target cells blocked LAK-induced

DNA fragmentation (175). On the other hand, CTL-mediated apoptosis does not require macromolecular synthesis (91). Blocking protein synthesis in P815 cells with cycloheximide or emetine did not inhibit CTL-mediated DNA fragmentation and apoptosis (36). Fragmentin-induced DNA damage was not inhibited in cycloheximide-treated YAC-1 cells, indicating that fragmentin-mediated apoptosis is not dependent on protein synthesis (146). However, it is unclear whether NK granule-mediated DNA fragmentation in 10T1/2 requires RNA and protein synthesis.

While CTLs and NK cells produce DNA fragmentation, there are conflicting reports whether the granules of these effectors are the mediators of DNA damage. Some investigators have found that CTL granules do not produce DNA fragmentation (39,52); others demonstrate that CTL granules degrade DNA (119). NK cell-derived granules induce DNA fragmentation, and Shi recently isolated a DNA fragmenting cytotoxin from NK cell granules (146). Whether lytic granules damage DNA or not, DNA fragmentation is not produced by cytolysin or perforin (4,38,119,146).

TNFα induces DNA fragmentation in susceptible target cells but as described previously, TNFα has not been identified within NK cell granules (72), and it seems to be unlikely that DNA degradation in 10T1/2 results from NK cell-associated TNFα. The TNFα-like cytotoxins

described above fragment the DNA of susceptible target cells (18,86). However, there is no evidence that NKCF or any other TNFα-like cytotoxin exists within the lytic granules of NK cells. Furthermore, DNA fragmentation of YAC-1 by fragmentin was not inhibited by antibodies against TNFα or TNFβ (146). This suggests that fragmentin is not immunologically or structurally related to TNFα.

NK cell granules contain proteases such as serine esterases (153,166). It is possible that serine esterases induce DNA fragmentation in target cells, and evidence is provided by the results of Hayes et al (56). This group has found that granzyme A from CTL granules, in the presence of cytolysin, produced DNA fragmentation in EL-4 target cells.

Shi has recently isolated a 32 kD cytotoxin from rat NK cell granules (146). The cytotoxin, fragmentin, has the capacity to damage DNA of YAC-1 and other lymphoma cells. In YAC-1, fragmentin produces apoptosis which is characterized by extensive membrane blebbing and damage, chromatin condensation, and rapid fragmentation of DNA. Fragmentin-mediated DNA degradation in YAC-1 cells requires participation of cytolysin. The molecule lacks serological cross-reactivity to TNFα and lacks BLT esterase activity. Fragmentin also demonstrates sequence homology to serine esterases: to NK cell and T cell RNK-1 and to mouse CTL

granzyme B.

Since fragmentin is present in rat NK cell granules, and DNA fragmentation of 10T1/2 is mediated by rat NK cell-derived granules, fragmentin may be the cytotoxin producing 10T1/2 DNA fragmentation. However, there are some problems with this possibility. Cytolysin is not required for 10T1/2 DNA damage because removing cytolysin activity from the granules did not inhibit DNA degradation. It is unclear why DNA disruption in 10T1/2 does not depend upon cytolysin but in YAC-1, cytolysin is absolutely required. Perhaps fragmentin induces DNA damage in 10T1/2 but through a mechanism different from that for YAC-1 cells. Some preliminary observations from our laboratory demonstrated that fragmentin could not disrupt the DNA in 10T1/2 cells. These observations could be either the result of different assay techniques, or perhaps 10T1/2 are not susceptible to fragmentin but to another, yet undisclosed DNA fragmenting cytotoxin.

As outlined above, the association between loss of adhesion and DNA fragmentation in 10T1/2 is unknown. Several observations indicate that serine esterases have a role in cytolysis. RNKP-1 in NK granules has been found to alter the adhesion properties of rodent tumor cells (136). CTL-associated granzyme A causes DNA damage in EL-4 (56). Fragmentin, which induces DNA damage, is

homologous to serine proteases from NK and CTL granules (146). Since serine esterases have been found to cause both cell adhesion changes and DNA fragmentation, it seems possible that some serine protease in NK granules is responsible for both loss of adhesion and DNA fragmentation in 10T1/2.

While the identity of the NK cell granule cytotoxin that mediates loss of adhesion and DNA fragmentation in 10T1/2 fibroblasts remains unknown, it is clear that the cytoplasmic granules produce this damage. It is also clear that stimulating proliferation in 10T1/2 fibroblasts with bFGF augments granule-mediated loss of adhesion and DNA degradation. The relationship between bFGF-stimulated proliferation in 10T1/2 fibroblasts and enhanced NK cell- or granule-mediated cytolysis remains unanswered. The bFGF-induced lesion in these cells is not known and can only be evaluated with the evidence presently available. Only the target 10T1/2 cells and not the NK cells are exposed to bFGF, and isolated granules have the capability to preferentially lyse bFGF-stimulated 10T1/2. Moreover, cold target inhibition assays have shown that NK cells do not preferentially recognize and bind to bFGF-stimulated, proliferating 10T1/2 cells (72). This suggests that the growth factor does not alter the activity level and cytolytic capability of the NK cell, but influences the

target cells' response to the cytotoxic granule molecules.

Basic-FGF initiates numerous activities in responsive cells and one or more such events may make 10T1/2 fibroblasts more susceptible to the NK cell's lytic apparatus. Basic-FGF is a growth and differentiation factor for mesodermal and neuroectodermal cells (19,47,48,49). The events bFGF initiates in responsive cells include: activating the synthesis of certain proteins and macromolecules; stimulating certain cellular genes, including oncogenes; inducing a transformation-like phenotype in responsive cells; stimulating DNA synthesis and mitosis; modifying the morphology, differentiation and senescence of cells. Thus, the bFGF-modified event which alters 10T1/2 susceptibility to NK-cytolysis may involve one, or more such cellular activities.

The cellular factor that renders bFGF-stimulated 10T1/2 cells more NK sensitive may be related to an event induced by the growth factor. Since bFGF stimulates proliferation in responsive cells, the bFGF-induced event may be related to some effect produced by proliferation and cell growth. If cellular proliferation is sufficient to make 10T1/2 more susceptible to granule-mediated lysis, then other factors that stimulate proliferation may similarly create this susceptibility. EGF, like bFGF, induces proliferation in responsive cells (25). If

proliferation is sufficient to augment NK sensitivity, then EGF-stimulated 10T1/2 cells should exhibit increased NK sensitivity. Khalil has found that EGF also increased the susceptibility of 10T1/2 cells to NK-mediated cytolysis (72). TGFβ inhibits cellular proliferation and is known to antagonize bFGF-mediated actions in responsive cells (49,100). Treating 10T1/2 with TGFβ produced a depression in the cells' NK sensitivity and this may have occurred through inhibition of 10T1/2 proliferation (72). However, EGF and TGFβ modify more than proliferation in responsive cells, and all cellular events produced by EGF and TGFβ are not known. It is unclear whether bFGF- and EGF-augmented NK sensitivity is simply the result of cell proliferation in 10T1/2 or the result of some other event, such as induction of a gene. It is also unclear whether the TGFβ-mediated decrease in NK sensitivity is related to TGFβ's inhibition of proliferation, since it has not been established that TGFβ inhibits proliferation in 10T1/2. Multifunctional EGF and TGFβ may alter NK sensitivity of 10T1/2 by influencing some function in the cells other than cellular proliferation.

Actively proliferating cells generally express increased rates of transcription and translation, while RNA and protein synthesis in quiescent cells is low (2). Basic-FGF stimulation induces in responsive cells the

transcription of certain genes, for example c-fos and c-myc. Additionally, bFGF induces synthesis of cellular proteins, such as fibronectin, plasminogen activator, thiol-dependent cathepsin and laminin (20,21,46,49). If active RNA and protein synthesis in target cells is an important parameter for NK-mediated cytolysis, then bFGF-stimulated 10T1/2 may experience augmented lysis as a consequence of the increased macromolecular synthesis. As detailed above, Zychlinsky et al have reported that active transcription and translation within the target cells may be necessary for LAK cell-mediated DNA fragmentation (175). Blocking these cellular processes with the drugs puromycin, emetine or actinomycin D inhibited LAK cell-mediated DNA fragmentation. On the other hand, other investigators have demonstrated that target cell transcription and translation are not necessary for CTL- or NK-mediated cytolysis (91). P815 cells exhibited CTL-mediated DNA fragmentation whether the cells were pretreated with or without the protein synthesis inhibitors cycloheximide or emetine (36). NK-mediated cytolysis of K562 cells was not diminished by inhibitors of protein synthesis such as cycloheximide and actinomycin D, and cytolysis occurred independent of K562's position in the cell cycle (35,77). Additionally, actinomycin D-pretreated L929 cells are more susceptible to lysis by TNFα than untreated L929 (94,95).

Actinomycin D is thought to inhibit RNA synthesis (3) and blocking transcription appears to enhance L929 cells' susceptibility to cytolysis. These observations indicate that RNA and protein synthesis probably affect cytolysis of only certain target cells, and may not fully account for the mechanism of NK sensitivity. According to these observations, it remains unclear whether RNA and protein synthesis are important influences on bFGF-augmented cytotoxicity against 10T1/2 cells.

While transcription and translation appear to have inconsistent effects on target cell sensitivity, other cellular activities occurring during proliferation may influence lytic susceptibility. In responsive cells, bFGF stimulates DNA synthesis and replication, and induces expression of specific genes. Basic-FGF-stimulated 10T1/2 demonstrate greater DNA synthesis than quiescent 10T1/2, an effect that has been illustrated by the increased ^{3}H-thymidine or ^{125}I-IUdR uptake by bFGF-stimulated but not by the unstimulated 10T1/2 (72). As described earlier, bFGF induces expression of the genes c-fos and c-myc (49). Since DNA fragmentation is an important mechanism of 10T1/2 cytolysis and since bFGF stimulation increases DNA fragmentation in 10T1/2, bFGF-induced DNA synthesis or gene expression may be responsible for the DNA degradation. DNA carries endonuclease hypersensitive sites or regions of

DNA that are preferentially digested by endonucleases such as DNase I (2). Endonucleases, whether they originate from the target cell or effector cell, are thought to mediate DNA fragmentation by CTLs and NK cells (91,146). The nuclease hypersensitive sites, associated with gene regulation, are located within nucleosome-free sites in active genes where transcription begins (2). If bFGF-stimulated 10T1/2 undergo greater DNA synthesis and gene expression than quiescent 10T1/2, then bFGF-treated cells may possess more nuclease hypersensitive sites in their DNA, making these cells more susceptible to DNA damage by endonucleases. However, there is scant evidence that associates DNA activities, such as replication and gene induction, with susceptibility to NK-mediated cytolysis. In TNF -mediated DNA fragmentation, L929 cell death was associated with cell cycle events, particularly the mitotic phase, but DNA fragmentation occurred whether DNA synthesis was active or inhibited (30). Blocking DNA synthesis in L929 cells did not prevent TNFα-mediated DNA degradation.

Basic-FGF induces a transformation-like phenotype in responsive cells (47,48,49). This may be a mechanism that leads to bFGF augmented NK-sensitivity of 10T1/2. Transformation of baby hamster and baby rat kidney cell lines with the adenovirus E1A gene increased the NK-mediated cytolysis of these target cells (32). The Ha-<u>ras</u> and

Ki-ras oncogenes transform cells, such as 10T1/2 and Rat-1 fibroblasts (40,51,64). The ras-transformed cells became more susceptible than the untransformed cells to NK-mediated cytolysis, and NK sensitivity increased with increasing ras gene expression. However, in ras-transformed Rat-1 cells, the oncogene seemed to increase the ability of the NK cells to recognize the transformed targets (64), an effect that does not occur for NK cells. NK cells did not preferentially recognise and bind to bFGF-stimulated 10T1/2 (72). Finally, since ras-transformed NIH 3T3 fibroblasts did not experience augmented cytolysis by NK cells, ras-induced NK sensitivity appears to occur independent of a transformation event (51).

Several investigators have reported that bFGF induces or enhances the synthesis of cellular proteins and macro-molecules, and it seems to modify the acute and long-term growth and morphology patterns of the responding cells (47,48,49). Basic-FGF alters the synthesis and secretion of the extracellular matrix components: collagen, fibronectin, laminin, proteoglycans. In bFGF-stimulated cells, the growth factor also modifies the expression of cellular cytoskeletal components such as actin, and creates changes in membrane fluidity and ruffling activity which reduces the cells' substratum adherence properties (48,49). Alterations in the cell cytoskeleton may explain

why bFGF-stimulated 10T1/2 are more susceptible to NK granule-associated loss of adhesion and DNA fragmentation. Modifications in the cytoskeletal components of 10T1/2 may alter the cells' adhesion properties and render 10T1/2 more susceptible to granule-induced loss of adhesion. Furthermore, recent observations have linked cytoskeletal changes, changes in actin microfilaments in particular, with sensitivity to TNFα-mediated DNA fragmentation (137). Human recombinant TNFα produced DNA fragmentation in C3HA cells treated with cycloheximide, and the DNA damage was associated with dissolution of the target cells' microfilaments. Cytochalasin E, which disrupts microfilaments, also resulted in TNFα-mediated DNA fragmentation in the normally insensitive C3HA cells. On the other hand, demecolcine, which disrupts microtubules and not microfilaments, did not lead to TNFα-mediated DNA fragmentation. These observations suggest that disruption of target cell microfilaments and cytoskeleton may make these normally resistant cells susceptible to TNFα-mediated DNA fragmentation. Whether the cytotoxin that damages 10T1/2 is related to TNFα or not, bFGF-induced cytoskeletal modifications in 10T1/2 may make these cells more susceptible to NK granule-mediated DNA fragmentation and loss of adhesion as well. It remains uncertain whether bFGF causes such effects in 10T1/2 cells, and whether

these effects increase NK susceptibility.

The oncogenes c-myc and c-fos regulate cellular proliferation and differentiation (20,49). Since bFGF has been found to induce the expression of both c-myc and c-fos (49), and because of their role in cellular proliferation and differentiation, expression of these oncogenes may influence NK sensitivity of 10T1/2 cells. Moreover, recent investigations have associated c-myc expression with NK cell susceptibility in both human and murine target cells (71,159,160). Inducing high levels of c-myc in human melanoma cells greatly augmented the target cells' sensitivity to NK mediated cytolysis: there was a strong relationship between c-myc expression and NK sensitivity. The mechanism for this effect is thought to be related to the expression of class I MHC molecules on the target cells: melanoma cells with low expression of MHC class I antigens were more susceptible to in vitro NK cytolysis, compared to cells with normal or high expression of class I antigens (71,160). In the melanoma cells, increased expression of the c-myc oncogene strongly down-regulated expression of MHC class I antigens, in particular, c-myc preferentially suppressed HLA-B (159,160). If bFGF increases c-myc expression in 10T1/2 fibroblasts, a similar depression in MHC class I antigen expression may occur in 10T1/2, rendering these cells more susceptible to NK-

mediated cytolysis. However, the relationship between the expression of c-myc, the expression of class I antigens and bFGF-stimulation in 10T1/2 cells remains unclear.

In conclusion, NK cell granule cytotoxins induced loss of adherence and DNA fragmentation but not membrane damage in 10T1/2 fibroblasts. The absence of membrane damage may specifically be the absence of extensive disruption of the cell membrane. Instead, granules may induce some minor membrane permeability changes which allow release of DNA fragments from the cells. DNA fragmentation and loss of adhesion appear to be major mechanisms of cell death, with more extensive membrane damage occurring secondarily. Additionally, since maximum DNA fragmentation occurs (in 4 hours) before maximum loss of adhesion occurs (18 hours), DNA damage may initiate 10T1/2 cell death, while loss of adherence is a secondary form of cell death. The exact nature of the lesion leading to loss of adhesion and DNA fragmentation is an area of future investigation.

Like 10T1/2 cytolysis with intact NK cells, granules caused augmented lysis of bFGF-stimulated, proliferating 10T1/2. This bFGF-augmented cytolysis was seen with the cytotoxicity adhesion and ^{125}I-IUdR release assays, but not with ^{51}Cr release assays. Loss of adhesion and DNA fragmentation, but not extensive membrane damage, seem to be the mechanisms responsible for bFGF-enhanced cytolysis.

Cytolysin, purified from NK cell granules, failed to produce loss of adhesion and DNA fragmentation in 10T1/2. Cytolysin also failed to mediate the bFGF-augmented lysis of these fibroblasts. Furthermore, cytolysin did not participate in 10T1/2 damage as a secondary, accessory cytotoxin, since eliminating cytolysin from granules did not alter the rate of cell death. While granule-associated cytolysin is not cytotoxic to 10T1/2, the responsible effector molecule(s) remains unidentified, and future research into this area may be undertaken.

The relationship between bFGF-stimulated proliferation in 10T1/2 and the augmented granule-mediated cytolysis of these target cells is unknown. Basic-FGF certainly induces many cellular events in responsive cells, with the possibility that one or more such events (occurring in 10T1/2) lead to this enhanced cytolysis. Along with the above questions, this question provides an area of future research.

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