THE ROLE OF $p21^{ras}$ IN NATURAL KILLER CELL FUNCTION

By

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ABSTRACT

Natural killer (NK) cells are a population of lymphocytes of large granular morphology. NK cells are able to kill sensitive targets without prior sensitization, in response to a network of activating and inhibitory signals transmitted by cellular receptors. The experiments presented in this thesis were designed to investigate the role of the $p21^{ras}$ protein in the function of NK cells, using as the model system the well described NK3.3 clone.

The farnesyl transferase inhibitor (B956) inhibited the NK3.3 mediated cytolysis by 50% at the concentrations used, a result compatible with an involvement of $p21^{ras}$ in NK cytotoxicity.

A number of G418 resistant clones derived from the NK3.3 cell line, transfected with the Ras^{Leu-61} mutant under the control of the rat metallothionein promoter were obtained. Stable cell lines expressing an activated mutant of Ras, Ras^{Arg-12}, were produced using a retroviral expression vector, which made this project possible. The expression of an activated Ras mutant increased NK3.3 mediated cytotoxic function, as measured against the NK-sensitive target K562, and the NK-resistant but lymphokine activated killer (LAK) sensitive cell line Raji. The increased cytotoxic activity of the cells expressing the activated Ras mutant against Raji suggests that Ras is involved in the IL-2 stimulation of NK cells to become LAK cells. Several levels of v-H-Ras mRNA were expressed by the NK3.3 cell lines, which differed in cytotoxicity. Furthermore, a spliced form of v-H-Ras mRNA was present only in the most cytotoxic cell line, indicating that different intracellular interactions with downstream effectors are possible.

Differences in the response to IL-2 of the NK3.3 cell lines transduced with the activated form of Ras were also observed. The v-H-Ras cells had a higher proliferative response when activated with IL-2 as compared to the controls, and IL-2 was required for optimal proliferation and cytotoxic function of the v-H-Ras transduced cell lines.

Furthermore, the involvement of Ras in the regulation of the expression of CD25 was a novel observation.

The expression of activated Ras induced at least a two fold increase in the IL-2 production in NK3.3 cells, in conformity with previous studies of T lymphocytes in the literature. The NK3.3R3 cell line, which expressed the lowest amount of v-H-Ras, secreted the highest amount of the cytokine. TNF and IFN production was not affected by the presence of activated Ras, suggesting that the Ras pathway, at the levels expressed by these cell lines, is not involved in the transcriptional activation of these genes. Expression of Ras^{Arg12} also correlated with lower conjugate formation frequency. The expression of activated Ras in NK3.3 affected the expression of several markers which are linked to an activated NK or mature functional phenotype. The percent positive cells for CD16, CD56 was increased in the Ras expressing cell lines, and variable effects on other molecules such as CD49d, e, and f, and CD71 were also observed. The expression of Ras^{Arg12} increased the BLT-esterase activity in NK3.3, but not the percent exocytosis. Others have shown that the activation of ERK2 is necessary for granule exocytosis following CD16 crosslinking in PBL-NK cells. However, the activation of Ras failed to increase this process, suggesting that the activation of Ras may be directly linked to the transcription of granzyme A and/or granzyme B genes, and thereby increasing the total intracellular content of the cytotoxic granules.

The experiments described in this thesis, especially the establishment and characterization of the NK3.3 Ras^{Arg12} cell lines, are the groundwork for the investigation of the $p21^{ras}$ protein in the function of natural killer cells. The results presented show that constitutive Ras activation is sufficient to maintain the cytotoxic phenotype of the cell lines under the conditions described, and opens new avenues of research in the effort to understand the signal transduction pathways which activate the cytolytic machinery of the NK cells.

DEDICATION

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LIST OF ABBREVIATIONS

[^{3}H]TdR	Tritiated thymidine
Ab	Antibody
ADCC	Antibody-dependent cell-mediated cytotoxicity
AP-1	Activator protein-1
ATCC	American Type Culture Collection
BCR	B-cell receptor
BLT	*N*-benzyloxycarboxy-L-lysine thiobenzyl ester
BSA	Bovine serum albumin
°C	Degrees Celsius
CD	Cluster of differentiation (marker)
cGy	CentiGray (rad)
cpm	Counts per minute
c-Ras	Cellular Ras
CSF-1	Colony stimulating factor-1
dATP	Deoxyadenosine triphosphate
DEPC	Diethylpyrocarbonate
DMEM	Dulbecco's modification of Eagle's medium
DMSO	Dimethyl sulphoxide
DNAM-1	DNAX accessory molecule-1
dNTP	Deoxyribonucleoside triphosphate
E/T	Effector to target
EDTA	Ethylenediamine tetraacetic acid
EGTA	Ethylene glycol bis-(β-aminoethylether)-N,N',N'-tetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
ERK	Extracellular signal-regulated kinase
FasL	Fas ligand
Fc	Immunoglobulin crystallizable fragment
FcεR	Receptor for the Fc portion of IgE
FcγRIIIA	Receptor IIIA for the Fc portion of IgG
FCS	Fetal calf serum
g	Gravity
GaLV	Gibbon ape leukemia virus
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GDP	Guanine diphosphate
GDS	Guanine nucleotide dissociation stimulator
GM-CSF	Granulocyte-macrophage colony stimulating factor
GTP	Guanine triphosphate
GTPase	Guanine triphosphatase
H-/K-/N-Ras	Harvey/Kirsten/N-Ras
HLA	Human leukocyte antigen
HRP	Horse radish peroxidase
ICAM (-1, -2, -3)	Intercellular adhesion molecule (-1, -2, -3)

ICE	Interleukin-1β-converting enzyme
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IL-2R	Interleukin-2 receptor
$IP_{(1,2,3,4)}$	Inositol (mono-, bis-, tris-, terakis-) phosphate
ITAM	Immunoreceptor tyrosine-based activatory motif
ITIM	Immunoreceptor tyrosine-based inhibitory motif
JAK	Janus kinase
KAR	Killer cell activatory receptor
KARAP/DAP12	Killer cell activatory receptor-associated protein
kb	Kilobase
kD	Kilodalton
KIR (-2D, -3D)	Killer cell inhibitory receptor (-2 or –3 intracellular domains)
LAK	Lymphokine-activated killer
LB	Luria Bertani (broth)
LGL	Large granular lymphocyte
LU	Lytic unit
LTR	Long terminal repeat
mAb	Monoclonal antibody
MAPK	Mitogen-activated protein kinase
MAPKK	MAPK kinase
MEK	MAPK/ERK kinase
MHC	Major histocompatibility complex
MLC	Mixed lymphocyte culture
MOPS	4-Morpholinepropanesulfonic acid
MSCV	Murine stem cell virus
MT	Metallothionein
neo	Neomycin resistance
NF-AT	Nuclear factor of activated T cells
NK	Natural killer
NK3.3R	NK3.3 Ras
NKRP-1	NK cell receptor protein-1
OD	Optical density (wavelength in subscript)
PAGE	Polyacrylamide gel electrophoresis
PBL	Peripheral blood lymphocytes
PBS	Phosphate-buffered saline
PBST	PBS with 0.5% Tween-20
PCR	Polymerase chain reaction
PGK	Phosphoglycerate kinase
PI-3 kinase	Phosphatidylinositol-3-kinase
PKC	Protein kinase C
PLA	Phospholipase A
PLC	Phospholipase C
PLD	Phospholipase D
PMA	Phorbol myristate acetate

PMSF	Phenylmethylsulfonyl fluoride
PTP	Protein tyrosine phosphatase
SD	Standard deviation
SDS	Sodium dodecyl sulphate
SG	Specific gravity
SH-2	Src homology-2
SHP (-1, -2)	SH-2 domain-containing tyrosine phosphatase (-1, -2)
SSC	Standard saline citrate
SSPE	Standard saline-phosphate-EDTA
STAT	Signal transducer and activator of transcription
Strip-1	STAT interacting protein-1
TAE	Tris-acetate-EDTA
TB	Terrific broth
TCR	T-cell receptor
TE	Tris-EDTA
TGF	Transforming growth factor
TIL	Tumour-infiltrating lymphocyte
T_m	Denaturing (melting) temperature
TNF	Tumour necrosis factor
VLA	Very late antigen
v-Ras	Viral Ras
x g	Times the force of gravity
ZAP-70	70kD zeta chain-associated protein

INTRODUCTION

Over the last decade, and especially over the past few years, a great deal of research has been done to investigate the molecular events triggered by the engagement of extracellular receptors. Engagement of these receptors leads to changes in the activation status of a variety of intracellular proteins, such as protein tyrosine kinases, serine/threonine kinases and lipid kinases. These, in turn, activate a variety of intracellular signals. It is clear that engagement of cell surface receptors leads to the activation of divergent signaling pathways, which result in the appropriate cellular response. One of the signaling pathways most extensively studied in this context is the pathway involving the GTP-binding protein, $p21^{ras}$. A variety of cellular receptors including T cell receptors (TCR), B cell receptors (BCR), and growth factor receptors, activate $p21^{ras}$. Despite the considerable amount of research done on this protein, the precise biological effect of $p21^{ras}$ activation in lymphocytes in general, and in natural killer (NK) cells in particular, is still not fully understood. This project's main objective was to investigate the signals that are generated downstream of $p21^{ras}$ as they relate to various aspects of the natural killer cell response, focusing on cytotoxicity, cytokine production, and marker expression.

The Natural Killer Cell

Natural killer (NK) cells constitute a subpopulation of lymphocytes that are distinct from B cells and T cells. NK cells are of large granular morphology, and are capable of recognising and lysing virally-infected and tumour cells without prior sensitization (Pross and Jondal, 1975; Jondal and Pross, 1975). Based on the phenotypic differences between NK cells and T cells, a NK clone referred to as NK3.3 was cloned by Kornbluth *et al.* (1982). This cell line was established *in vitro* by cloning primary mixed lymphocyte culture (MLC)-activated peripheral blood lymphocytes (PBL) in soft agarose.

It kills the NK-susceptible target cell line K562, and lacks T cell or monocyte markers. NK3.3 was found to be morphologically and cytochemically similar to LGL. It is interleukin 2 (IL-2) dependent for growth, and since its establishment *in vitro* it has been used for the study of NK cells by many laboratories world-wide, and has been accepted as a true NK cell clone.

The role of NK cells in the immune system as part of the first line of defence against viral infection, malignancy and metastasis formation has become evident (Pross and Baines, 1976; Pross and Baines, 1982; Pross, 1986; Trinchieri, 1989; Lanier and Phillips, 1992; Pross and Lotzova, 1993; Lopez *et al.*, 1993). The NK cell's importance to the well-being of the organism is reinforced by the fact that NK deficiencies are rare and, when present, the patient is overwhelmed by life-threatening viral infections (Biron *et al.*, 1989). This phenomenon is not surprising, as NK cells activate other arms of the immune system via secretion of an array of cytokines upon activation, such as IL-2, IL-5, IFN, TNF, GM-CSF, CSF-1, IL-12, and IL-10 (Trinchieri, 1989; Yoshihara *et al.*, 1993; Jewett and Bonavida, 1994; Kubin *et al.*, 1994; Warren *et al.*, 1995; Mehrotra *et al.*, 1998). By the secretion of these cytokines NK cells recruit other arms of the immune response to the site of infection or tumour growth, and may affect haematopoiesis. It has been clearly established that NK cells are of lymphoid origin and that T cells and NK cells may develop from a common progenitor (Lanier *et al.*, 1992; Georgopoulos *et al.*, 1994). Although NK and T cells express several common markers, unlike T cells NK cells do not undergo a productive T cell receptor (TCR) gene rearrangement (Biassoni *et al.*, 1993). However, the CD3ζ chain, which is associated with the TCR, is often found associated with the Fcγ IIIA receptor (CD16) expressed on NK cells (Lanier *et al.*, 1989). Despite these similarities, there is a marked difference between NK and T cells. Since NK cells do not express a functional TCR, NK target recognition and subsequent activation must be fundamentally different from that of cytotoxic T lymphocytes. Our current understanding is that NK-mediated cytolysis is a sensitive balance between activation and

inhibitory signals, which results in target killing and cytokine production. The activation of the receptors, whether inhibitory or activating, trigger an intracellular network of phosphorylation and dephosphorylation mediated by kinases and phosphatases which subsequently results in the proper cellular response. In recent years, there has been amazing progress in our understanding of NK target recognition, and it has become clear that NK activation is linked to MHC expression on the target cell surface (Kaufman *et al.*, 1993).

Killer Cell Inhibitory Receptors (KIR)

Three types of inhibitory receptors which bind MHC class I have been identified on NK cells of human and murine origin. They bind different components of MHC on the target cells although, surprisingly, they do not share any homology. When these receptors engage, inhibitory signals are transmitted that completely abolish NK cytotoxicity directed at the target expressing the corresponding MHC (Vitale *et al.*, 1995; D'Andrea *et al.*, 1995; Lanier *et al.*, 1995; Colonna and Samaridis, 1995; Vitale *et al.*, 1996).

The mouse receptors for MHC, collectively referred to as Ly-49 (the Ly-49A-I), belong to the C-type lectin family, and map to Chromosome 6 in a region called the "NK gene complex" (Yokoyama and Seaman, 1993). The Ly-49A molecule is present on 10-20% of murine NK cells and binds to H2-D^d, Ly-49C binds to H2-K^b and H2-$D^{d,b}$, and Ly-49G2 may have H2-D^d and H2-L^d specificity (Daniels *et al.*, 1994; Mason *et al.*, 1995; Yu *et al.*, 1996; Brennan *et al.*, 1996;). Nearly 80% of the NK cells express one of these receptors, and one NK subset can express more than one type of Ly-49 molecule. Furthermore, it has been demonstrated that Ly-49 undergoes allelic exclusion without gene rearrangement, and that NK cells can express more than one Ly-49 receptor (Held *et al.*, 1995). It has also been proposed recently that the MHC environment in which NK cells mature, and the presence of MHC deficient cells, have a role in the frequencies and levels of expression of the different Ly-49 molecules, dictating self-tolerance (Dorfman

and Raulet, 1996). Similar to T cells, an educational process seems to occur that limits the number of NK cells coexpressing multiple Ly-49 receptors (Held and Raulet, 1997). Most Ly-49 type receptors contain an immunoreceptor tyrosine-based inhibitory motif (ITIM). Following ligation to the corresponding MHC class I, the receptor is phosphorylated and recruits to the membrane Src homology-2 (SH2) domain-containing tyrosine phosphatases (SHP) 1 or 2, which inhibit NK mediated cytotoxicity (Mason *et al.*, 1997).

In humans, several killer inhibitory receptors (KIRs) have been described. The killer cell inhibitory receptor family was mapped to Chromosome 19q13.4 (Wilson *et al.*, 1997). Unlike the mouse receptors, they are type I glycoproteins which contain 2 or 3 Ig-like domains (KIR2D or KIR3D) in the extracellular region of the receptor. Several proteins that belong to the p58 and p70 family of proteins have been isolated, showing a heterogeneous population of receptors that may bind with different affinities to the different HLA alleles (D'Andrea *et al.*, 1995; Colonna and Samaridis, 1995). The inhibitory receptors are coexpressed on NK cell clones in different combinations, conferring allospecificity, and are functionally independent; *i.e.*, cross-linking of one receptor results in complete inhibition of lysis of sensitive targets of the corresponding MHC only (Vitale *et al.*, 1995; D'Andrea *et al.*, 1995; Lanier *et al.*, 1995; Colonna and Samaridis, 1995; Vitale *et al.*, 1996). The p58 family of proteins, described by Moretta's group (Ciccone *et al.*, 1992), corresponds to proteins of 55 kD and 58 kD that are expressed on the cell surface as monomers or noncovalently linked dimer glycoproteins of 58 kD or 55 kD alone or in combination. Functional studies using cw3-transfected target cells and antibody masking suggest that p58 is a receptor specific for HLA-C which transmits inhibitory signals (Moretta *et al.*, 1993). The p58.1 KIR is a receptor containing two Ig- domains which recognizes HLA-C with asparagine at position 77 and lysine at position 80. The 58.2 KIR recognizes HLA-C alleles with serine at position 77 and asparagine at position 80 (Winter and Long, 1997). Also, a role for HLA-G in

inhibiting subsets of NK cells expressing the p58 receptors has been demonstrated in reproduction (Pazmany *et al.*, 1996). Substantial evidence shows that the α_1 domain of the HLA heavy chain is crucial for the recognition by KIRs. Similar to the alpha-beta TCR, these receptors seem to recognize the peptide binding groove of the MHC-I, but the presence of the peptide in the MHC binding groove does not seem to be needed for the recognition (Malnati *et al.*, 1995; Correa and Raulet, 1995; Kurago *et al.*, 1995; Biassoni *et al.*, 1995; Luque *et al.*, 1996). However, the binding of p58 to HLA-cw4 shows peptide specificity (Rajagopalan and Long, 1997). Winter and Long (1997) have shown that one amino acid difference in the p58 molecule is sufficient to distinguish between different HLA-C allotypes. The human NK inhibitory p58 and p70 receptors have been characterized, cloned and sequenced.

Lanier *et al.* described a second receptor of 70 kD which binds HLA-B alleles (Litwin *et al.*, 1994; Lanier *et al.*, 1995), and a 70 kD receptor specific for HLA-A3 was first described by Collona's group (Dohring and Colonna, 1996). The KIR p70 is a three Ig-domain containing receptor which recognizes the HLA-Bw4 motif. The recognition occurs in the α_1 helix of the HLA-B heavy chain, within the residues 77-83 (Gumperz *et al.*, 1997). The presence of a peptide in the MHC groove is important for the recognition of the MHC molecule by the receptor, however it does not confer its specificity (Malnati *et al.*, 1995).

In addition to KIR an additional type of receptor is responsible for MHC recognition. In humans, similar to the mouse MHC receptors, lectin type receptors have been found. These receptors are NKR-P1 (NK cell receptor protein-1), NKG2A - NKG2E, the CD94 proteins and CD69 (Duchler *et al.*, 1995; Ryan *et al.*, 1995; Plougastel and Trowsdale, 1997; Carretero *et al.*, 1997; Perez-Villar *et al.*, 1997). The receptor CD94/NKG2 is a heterodimer, consisting of the glycoprotein CD94 that is bound to NKG2A, B or NKG2C. All three units have been mapped to Chromosome 12 at locus p12.3-p13.1 in humans, and the mouse CD94 is located on Chromosome 6 (Vance *et al.*,

1997). Unlike the other KIRs the CD94 is expressed on all NK cells, and therefore it may be a more general inhibitor receptor (Lopez-Botet *et al.*, 1997). Recently Braud *et al.* (1998) showed that the complex CD94/NKG2 binds to the non-classical MHC molecule HLA-E. NKG2A and NKG2C differ in the intracellular domain NKG2A possesses an ITIM while the NKG2C does not, while exhibiting a charged amino acid (Cantoni *et al.*, 1998).

The cytoplasmic domains of KIRs are heterogeneous. Some isoforms contain long cytoplasmic tails, which contain ITIM. Similarly to the mouse Ly-49 receptors, the ITIM recruits protein tyrosine phosphatases such as PTP1C, PTP1D, SHP-1 and SHP-2, upon receptor phosphorylation, by the lck tyrosine kinase (Biassoni *et al.*, 1996; Vely *et al.*, 1997). These phosphatases, which belong to the SH2 domain-containing family of phosphatases, are expressed uniquely in the haematopoietic system, and are involved in the regulation of the BCR, TCR and a variety of cytokine receptors (Hunter, 1995). Interestingly, inhibition of signals from the TCR by KIR expressed on T cells via PTP1C recruitment also has been reported (Fry *et al.*, 1996). Furthermore, inhibition of NK activation via CD16 crosslinking by KIR has also been shown. This inhibition is entirely dependent on SHP–1 since expression of an inactivated dominant mutant reverses the inhibitory effect of KIR ligation (Binstadt *et al.*, 1996). The nature of the downstream effectors, which are dephosphorylated/functionally inhibited, is not yet known. The presence of SH2 domains allows these phosphatases to interact with a variety of proteins that interact with the Src family of protein tyrosine kinases such as $p56^{lck}$, $p59^{fyn}$, $p62^{yes}$, and $p60^{src}$. SHP-1 has been shown to interact with the kinase ZAP-70, but this cannot acount on its own for the inhibition of the killing (Binstadt *et al.*, 1996; Plas *et al.*, 1996). Future research will reveal which substrates are dephosphorylated in NK cells and the signaling pathways involved. Despite significant progress in our understanding of NK inhibition by the interaction between KIRs and MHC, the mechanisms and receptors that render NK capable of cytolysis are still poorly understood.

NK Cell Activation

In the search for the NK-activating receptor, few moieties expressed on NK cells have been found to trigger NK cytotoxicity when bound to their counter receptors. One such receptor, NK receptor protein-1 (NKR-P1), was originally described in rat NK cells, and belongs to the lectin family (Giorda *et al.*, 1990). Cross-linking of this molecule induces cytotoxicity and IFNγ production in murine NK cells (Giorda *et al.*, 1990; Arase *et al.*, 1996). There are three homologues of the NKR-P1 in the rat which can transmit either inhibitory or activatory signals (Al-Aoukaty *et al.*, 1997). In humans, only one such receptor has been described, CD161 (Arase *et al.*, 1997). In addition, two members of the p58 group of molecules involved in HLA recognition, p50 and p50.3/PAX, were identified on some NK subsets (Olcese *et al.*, 1997; Bottino *et al.*, 1996). These molecules are highly homologous to the p58 inhibitory receptors, but differ in their cytoplasmic domains, and therefore transduce different intracellular messages. Unlike their inhibitory analogues, these molecules redirect killing, and enhance production of IFNγ and TNFα (Bottino *et al.*, 1996; Biassoni *et al.*, 1996). Recently, it has been shown that the p50 molecules are a truncated form of KIR which has a short cytoplasmic tail (which does not possess an ITIM), two extracellular Ig-domains which bind to HLA-C, and a positively charged residue in the intracellular domain (Biassoni *et al.*, 1996). The lack of an intracellular kinase domain suggests that the receptor must interact with kinases similar to the cytokine receptors. Recently two proteins have been shown to associate and become tyrosine phosphorylated, therefore being able to act as the dock for other protein kinases to associate and transmit their signals. One is a p13 protein, referred to as KARAP which becomes phosphorylated and interacts with the KAR p50.1 (Campbell *et al.*, 1998). Recently, Lanier *et al.* (1998) have shown that the truncated KIR associates with DAP12 or KARAP, which contains an immunoreceptor tyrosine-based activatory motif (ITAM), and therefore is able to transmit positive signals. More recently, a novel triggering molecule, NKp44, has been described by Vitale *et al.*(1998). This

molecule is expressed selectively on activated NK cells, and is involved in non-MHC restricted lysis. Furthermore, NKp44 is bound to KARAP/DAP12 and therefore able to transmit activatory signals mediated by tyrosine phosphorylation. FcγRIIIA (CD16), which binds to antibody-coated targets, is widely accepted as a NK cell activator molecule, and plays a role in its regulation. The cross-linking of this molecule activates the transcription of IFN, TNF, GM-CSF, M-CSF and IL-8, possibly via activation of the family of transcriptional factors belonging to the nuclear factor of activated T cells (NFAT) family of proteins (Aramburu *et al.*, 1995; Trinchieri and Valiante, 1993). A recent report shows that incubation of NK cells with monomeric IgG in the presence of IL-2 up-regulates the expression of the IL-2R α chain, and cytokine production (Sulica *et al.*, 1996). The binding of the effector cell to the target cell involves a number of adhesion molecules, which also transmit co-stimulatory signals. Some of these molecules may even trigger the cytolytic cascade. For example CD2 and CD16 co-stimulation enhances NK cytotoxicity and cytokine production, and blockage of CD2 with antibodies inhibits NK cytotoxicity to some degree, but does not block it entirely (Vivier *et al.*, 1991). Co-stimulatory signals from molecules such as CD28, CD44, CD45, integrins, CD27, and, recently, CD40, seem also to be required for optimal NK function and proliferation (Tan *et al.*, 1993; Sconocchia *et al.*, 1994; Galandrini *et al.*, 1994; Nandi *et al.*, 1994; Shen *et al.*, 1995; Xu and Chong, 1995; Pignata *et al.*, 1995; Yang *et al.*, 1996; Carbone *et al.*, 1997). A molecule named DNAM-1 has also been described, and it has a stimulatory effect on NK cells (Shibuya *et al.*, 1996). The other group of adhesion molecules which seem to have an impact on effector target interactions and may even transmit activatory signals into the cell are the β_2 integrins. Cross-linking of CD11a/CD18, which binds ICAM, induces calcium influx, phosphoinositide turnover, and protein phosphorylation (Poggi *et al.*, 1996). In addition NK cells can be activated via ligation of CD11b/CD18 (Sugie *et al.*, 1995).

Mechanisms of NK Cell-Mediated Cell Death

Similar to cytotoxic T cells, NK cells are capable of killing altered cells via exocytosis of granules containing lytic mediators. Within a few minutes after conjugate formation, the intracellular granules become reoriented toward the target (Geiger *et al.*, 1982). Then, the granules are secreted into the intercellular space, followed by lesion formation and death of the target either by apoptosis or necrotic death (Trapani and Smyth, 1993; Yamauchi *et al.*, 1996; Roger *et al.*, 1996). One of the proteins present in the granules is perforin which, as the name indicates, is a pore forming protein similar to the C9 component of the complement system (Tschopp *et al.*, 1986). Perforin can bind to various lipid molecules in the membrane in the presence of calcium (Tschopp and Jongeneel, 1988). Following the attachment to the membrane, a conformational change occurs, which enables the protein to insert into the lipid layer (Liu *et al.*, 1995). NK cells express perforin constitutively, and synergy between perforin and other granule components (granzyme B) is required for the apoptotic effect (Shi *et al.*, 1997). The other components of the granules released by NK cells are the granzymes, which are also constitutively expressed by NK cells, but their expression can be upregulated by cytokine stimulation (Ojcius *et al.*, 1991; Ebnet *et al.*, 1995). Granzyme A and granzyme B are serine proteases, and comprise about 90% of the protein inside the cytotoxic granules (Henkart *et al.*, 1987; Ojcius *et al.*, 1991). Their *in vitro* activity is assessed by hydrolysis of synthetic thiobenzyl ester substrates. Granzyme B, which is the most abundant of the two, does not induce apoptosis when the cells are exposed to it unless perforin is present (Jans *et al.*, 1996; Shi *et al.*, 1997). It has been shown that perforin does not only provide the route of entrance for the granzymes, since permeabilization of the membrane by other means in the presence of granzyme B did not cause apoptosis of the targets (Shi *et al.*, 1997). In the presence of perforin both granzyme A and B undergo nuclear localization (Trapani, 1998). However granzyme B is more potent in the onset of apoptosis than granzyme A, and its kinetics for localization to the nucleus are slower (Shiver *et al.*,

1992; Shi *et al.*, 1992). Granzyme A has been shown to have interleukin-1β converting enzyme (ICE)-like activity in that it can cleave pro IL-1 (Irmler *et al.*, 1995). However the mechanism by which this enzyme causes apoptosis is uncertain, since it hasn't been shown to activate any of the caspases known to be involved in the process (Trapani, 1998). However, granzyme B is able to activate many of the caspases that are involved in the apoptotic cascade (Van de Craen *et al.*, 1997). In addition, it has been shown that granzyme B on its own is able to reach the nucleus and cleave nuclear substrates directly (Garner *et al.*, 1994). Chymases, enzymes with a chymotrypsin like specificity, are also of importance in pore formation by perforin, and recently chymase I has been purified (Woodward *et al.*, 1998). Another mechanism by which NK cells are able to induce apoptosis in the target cell is by the interaction of Fas with FasL, which is also expressed by NK cells (Oshimi *et al.*, 1996). Ligation of the FasL to Fas engages the cell into the apoptotic cascade, leading to activation of caspases, and DNA fragmentation (Montel *et al.*, 1995; Medvedev *et al.*, 1997). This pathway is entirely caspase dependent, and therefore it implies a different molecular mechanism than the one mediated by the granzymes (Longthorne and Williams, 1997).

Signal Transduction from the IL-2 Receptor, the Ras Pathway and the JAK-STAT Pathway

Cytokines such as IL-1, IL-4, IL-7, IL-12, IL-15, transforming growth factor β1 (TGFβ1), TNF, IFN, and stem cell growth factor are important for NK function and proliferation of both mature and progenitor NK cells (Kobayashi *et al.*, 1989; Hayakawa *et al.*, 1991; Robertson *et al.*, 1992; Miller *et al.*, 1993; Mason *et al.*, 1995; DeBlaker-Hohe *et al.*, 1995; Shibuya *et al.*, 1995). However, the most important factor for NK proliferation and activation is IL-2 (Trinchieri, 1989). IL-2 is a cytokine that, when bound to its ligand, generates a complex array of cellular responses both in NK cells and T cells. IL-2 is not only a growth factor for T cells and NK cells, but is also the

main cytokine which is able to activate NK cells to become lymphokine-activated killer (LAK) cells (Trinchieri, 1989). LAK cells differ from NK cells in the broader range of susceptible targets, expression of adhesion molecules and cytokine production, as reviewed by Frederick *et al.* (1997). It is interesting that binding of the IL-2 cytokine to its receptor is able to generate multiple cellular responses. Two major transduction pathways are activated by the IL-2 receptor, the Ras/Raf-1/mitogen-activated protein kinase (kinase) MAPKK/ mitogen activated kinase (MAPK) pathway, and the signal transduction pathway mediated by the JAK/STAT family of proteins. The IL-2 receptor is a heterodimeric receptor composed of the α, β, and γ chains, which form the high affinity IL-2 receptor (Kondo *et al.*, 1993). NK cells express the β and γ chains at all times, and upon activation the α chain is transcribed and, together with the other two chains which are constitutively expressed, the high affinity receptor is formed. The JAK (Janus kinase) family of kinases consists of three members JAK1, JAK2 and Tyk2, which were identified through cDNA-cloning approaches. The β chain of the IL-2R interacts with JAK1 and the γ chain interacts with JAK3 (Tanaka *et al.*, 1994; Russell *et al.*, 1994; Miyazaki *et al.*, 1994). Binding of IL-2 to its receptor results in rapid phosphorylation of JAK1 and JAK3 on tyrosine residues (Miyazaki *et al.*, 1994; Johnston *et al.*, 1994). The downstream substrates of the JAKs are the IL-2 receptor itself, the signal transducers and activators of transcription (STATs), and the adapter molecule Shc, which enables the receptor to interact with the Ras pathway (Chen and Chen, 1997). IL-2 also activates PI-3 kinase, through unknown mechanisms, in a MEK dependent fashion (Karnitz and Abraham, 1995). The best characterized substrates of JAKs are the STATs. The STAT family of proteins are transcription factors which are activated by tyrosine phosphorylation, after docking to the ligand activated receptor through its SH2 domain (Hemmann *et al.*, 1996). So far, eight genes encoding members of the STAT family of proteins have been identified (Darnell, 1997). The binding of IL-2 to its receptor, recruits to the membrane the STAT3 and STAT5 members of the family in activated peripheral

blood lymphocytes (Lanier, 1995). Recently, a protein named Strip-1 (STAT interacting protein) has been identified, which bridges between the JAKs and the STATs at the receptor site (Darnell, 1997). Upon tyrosine phosphorylation, the STATs form homodimers or heterodimers capable of nuclear translocation and activation of DNA transcription (Darnell, 1997). Interestingly, Ran, a GTP-binding protein, has been shown to be involved in the translocation of the dimers to the nucleus (Sekimoto *et al.*, 1996). In addition to the tyrosine phosphorylation these factors can be phosphorylated on serine/threonine residues as shown by point mutations, and by treatment of the STATs with phosphatases which impaired their activity (Sengupta *et al.*, 1996). Possible regulators of this phosphorylation are the MAPKs, which provide an additional "cross-talk" between the Ras pathway and the STATs (David *et al.*, 1995; Chung *et al.*, 1997). Recently, the phosphatase SHP-2 has been shown to positively regulate MAPK activity mediated by cross-linking of the IL-2 receptor, via association with Grb2 and possibly with PI-3 kinase (Gadina *et al.*, 1998). Downstream events of the STATs activation and translocation to the nucleus include activation of nuclear proteins such as Myc, Fos, Bcl2 and Bcl-X_L, which are important for T cell proliferation and prevention of apoptosis (Pellegrini and Dusanter-Fourt, 1997).

In addition to the activation of the JAK/STAT pathway, binding of IL-2 to the high affinity IL-2 receptor (IL-2R) results in accumulation of GTP-$p21^{ras}$ complexes in T cells and NK cells (Graves *et al.*, 1992; Galandrini *et al.*, 1996). This process is dependent upon protein tyrosine kinases, since the process is inhibited by herbimycin (Izquierdo and Cantrell, 1993). Shc is one of the adaptor molecules involved in Ras activation, as shown by the high levels of Shc tyrosine phosphorylation and the coprecipitation of Shc with the IL-2Rβ chain after stimulation with IL-2 (Ravichandran and Burakoff, 1994; Zhu *et al.*, 1994; Galandrini *et al.*, 1996). $p95^{vav}$, a protein expressed mainly in the haematopoietic system, that has a tyrosine kinase-modulated guanine nucleotide-releasing factor activity, is also rapidly phosphorylated in response to IL-2,

and has been shown to activate Ras (Evans *et al.*, 1993; Gulbins *et al.*, 1995). However, more recent studies showed that Vav is mainly a guanine-nucleotide exchange factor for Rac, and is involved in cytoskeleton formation (Han *et al.*, 1998). It is not clear yet which cellular responses mediated by IL-2 receptor crosslinking are regulated exclusively via the Ras pathway. Few reports in the literature address this question using mutants of Ras or of proteins downstream in the pathway. It has been shown by such studies that inhibition of Ras with the dominant negative mutant N-17 induced cell apoptosis in T cells and abrogated the proliferative response to IL-2 (Gomez *et al.*, 1996). It has also been shown that downstream effectors such as MEK1 and ERK are required for IL-2 transcription (Whitehurst and Geppert, 1996).

Signal Transduction by Triggering Molecules in NK Cells

Upon the engagement of these molecules on the cell's surface, resulting in the array of biological effects listed above, intracellular events occur which are initiated by a cascade of protein phosphorylation and lipid phosphorylation (Einspahr *et al.*, 1992; Liao *et al.*, 1993; Rabinowich *et al.*, 1994; Gismondi *et al.*, 1995; Xu and Chong, 1995; Rabinowich *et al.*, 1995; Teng *et al.*, 1996). CD16 was one of the first receptors identified to trigger NK cell-mediated cytolysis. Upon ligation of CD16, a rapid increase in concentrations of intracellular metabolites such as IP1-4, and Ca^{++}, as well as a cascade of phosphorylation occur (Liao *et al.*, 1993; Kanakaraj *et al.*, 1994). It has also been shown that the basal level of phosphorylation in NK cells is substantially higher than in resting T cells (McVicar *et al.*, 1996). Some of the proteins that are phosphorylated are the associated CD3ζ chain, phospholipase C (PLC 1 and 2), $p72^{syk}$, Shc, and p36 (Vivier *et al.*, 1991; Ting *et al.*, 1992; Liao *et al.*, 1993; Stahls *et al.*, 1994; Stahls *et al.*, 1994; Galandrini *et al.*, 1996). In addition, activation of protein kinase C and phosphatidylinositol-3 (PI-3) kinase occurs (Azzoni *et al.*, 1992; Ting *et al.*, 1992; Park *et al.*, 1993). Interestingly, it has been shown that granule release triggered by CD16

stimulation requires PI-3 kinase activation, but stimulation of NK cells by binding to sensitive targets requires PKC, suggesting that divergent signal transduction pathways are triggered by targets and CD16 (Bonnema *et al.*, 1995). Furthermore it has been recently shown that granule exocytosis triggered by CD16 cross-linking involves phospholipase A which is regulated by MAPK (Milella *et al.*, 1997). In addition, it has been shown that PI-3K activation mediated by CD16 cross-linking in NK cells involved the phosphorylation of Cbl, which interacts with Grb2, Shc, or p36 and the p85 subunit of PI-3K (Cerboni *et al.*, 1998).

Stimulation of NK cells via other receptors leads to similar events. Cross-linking of CD28 involves tyrosine phosphorylation and activation of PI-3 kinase and PKC, CD45 stimulates $p56^{lck}$ and tyrosine phosphorylation, and the $\alpha4\beta1$ and $\alpha5\beta1$ integrins stimulate tyrosine phosphorylation (Rabinowich *et al.*, 1995; Shen *et al.*, 1995; Xu and Chong, 1995; Teng *et al.*, 1996). In addition, it has been shown that CD2 activation of NK cells requires PI-3 kinase and is mediated by $p72^{syk}$ (Umehara *et al.*, 1997). A few studies suggest the involvement of the Ras-mediated signal transduction pathway in the activation of NK cells. First, a substantial number of the protein tyrosine kinases listed above activate $p21^{ras}$. Second, as described in more detail below, Ras is activated via the IL-2R and via CD16 cross-linking in NK cells (Galandrini *et al.*, 1996; Rodriguez-Viciana *et al.*, 1996).

The $p21^{ras}$ Protein

Many studies have placed Ras in a critical role in various cellular responses such as proliferation, differentiation, and transformation (Graves *et al.*, 1992; Schlaepfer *et al.*, 1994). Space restrictions limit this description to the lymphoid system, mainly to T cells, which are believed to be closely related to NK cells in ontogeny. Recent data suggest that similarities exist in the signalling pathways that regulate the responses mediated by the TCR in T cells and the KIRs or KARs in NK cells. Ras belongs to the GTPase extended

family of proteins. In mammalian cells, four Ras proteins are expressed: K-ras, H-ras and N-rasA and B. All these proteins are activated by binding to GTP, and inactive when bound to GDP (Boguski and McCormick, 1993). As a post-translational modification, a farnesyl isoprenoid lipid is added to the protein by the enzyme farnesyl transferase. This modification is required for the correct cellular localization of the protein, and for transduction of the intracellular signals (Omer *et al.*, 1997). The best-described signal transduction pathway from the activated $p21^{ras}$ to the nucleus involves the Raf protein family, and the mitogen-activated protein kinases (MAPK), which activate a variety of transcription factors (Moodie *et al.*, 1993; Hunter, 1995). In T cells, TCR and IL-2 ligation is coupled by Ras to the NFAT family of transcription factors, which regulate cytokine and chemokine gene expression such as IL-2 and IL-2R in T cells, and IL-5 and GM-CSF in mast cells (Rayter *et al.*, 1992; Turner and Cantrell, 1997). Recently the Raf-1/MAPKK-1/ERK-2 pathway has been shown to be necessary for lectin stimulation of the IL-2 gene transcription in T cells (Lafont *et al.*, 1998). Ras is also activated upon IL-2 receptor ligation, leading to T cell proliferation, and is involved in differentiation and expansion of $CD4^-CD8^-$ thymocytes (Izquierdo-Pastor *et al.*, 1995; Swat *et al.*, 1996). Studies using transgenic mice in which a dominant negative mutant of Ras was expressed in the T lineage under the control of the lck promoter (Swan *et al.*, 1995) showed that expression of this mutant substantially reduced the development of single positive $CD4^-CD8^-$ or $CD4^-CD8^+$ $CD3^{hi}$ from the double positive precursor, suggesting a role for $p21^{ras}$ in thymocyte repertoire selection. Anergic T helper cells are blocked in their ability to activate the Ras and MAPK pathway, and are defective in IL-2 production, but are able to secrete normal amounts of IFNγ. Interestingly, PMA and ionomycin can activate Ras in these cells, but this does not restore their ability to secrete IL-2 (Fields *et al.*, 1996). Furthermore, in T thymocytes, Ras is required for positive selection, and so is the Raf/MAPK signalling pathway, but the Raf/MAPK pathway is not required for T cell proliferation (Alberola-Ila *et al.*, 1995; Swan *et al.*, 1995). More evidence that the

Raf/MAPKK/MAPK pathway is not the only pathway activated by Ras was presented in studies on fibroblast transformation, that have concluded that this pathway is not sufficient for transformation mediated by oncogenic Ras (White *et al.*, 1995). Recently, several studies have linked Ras to other less well-defined signalling pathways. Ras activates phospholipase D via a Ral guanidine nucleotide dissociation stimulator (Ral-GDS)-like protein, which is linked to protein kinase C activation. Ras interacts directly with PI-3 kinase, an interaction that enhances its enzymatic activity (Rodriguez-Viciana *et al.*, 1996). Both PI-3 kinase and PKC are functionally coupled to antibody-dependent cell-mediated cytotoxicity (ADCC) and granule exocytosis by NK cells (Kanakaraj *et al.*, 1994). Furthermore, Ras is coupled to signaling pathways mediated by the Rho family of GTP-ases, such as RhoA, CDC-42 and Rac-1 (Alberola-Ila *et al.*, 1995; Prendergast *et al.*, 1995). Recently, cooperation of Syk and Rac-1 was reported in JNK activation in T cells (Jacinto *et al.*, 1998). The Rho family of proteins has been linked to cytoskeleton organization and cell polarity, and an increasing body of evidence suggests that this family of proteins may have an important function in mediating signals in the immune system (Ridley and Hall, 1992; Takaishi *et al.*, 1994; Kotani *et al.*, 1995). Recently, RhoA has been proposed to control the motility and cytotoxic function of NK cells (Lang *et al.*, 1996). In T cells, Rac-1 activation is sufficient for AP-1 regulation, but not for NFAT activation, which is mediated via the MAPK pathway (Genot *et al.*, 1996). In addition, using reporter gene constructs, Turner and Cantrell (1997) showed that Ras requires Rac-1 for the activation of NFAT by FcεR in mast cells. On the other hand, the same study concluded that activation of Ras only is sufficient for the activation of another transcription factor, Elk-1. These results place Rac-1 as a downstream effector of Ras for some cellular responses, similar to findings in fibroblasts (Alberola-Ila *et al.*, 1995). Finally, in NK cells, Ras is activated by CD16 cross-linking and by IL-2R ligation, and ERK-2 is required for CD16 mediated granule exocytosis (Milella *et al.*, 1997; Galandrini *et al.*, 1996). Both CD16 and IL-2R play a central role in NK function,

activation and proliferation (Trinchieri, 1989). Furthermore, CD28, CD45, integrins and cytokine receptors that upregulate NK function, have been shown to interact with components of the Ras signalling pathway, or directly activate Ras in NK cells or other systems (Lewis *et al.*, 1991; Nunes *et al.*, 1994; Montel *et al.*, 1995; Xu and Chong, 1995; Lee *et al.*, 1996). The signal transduction pathway mediated by Ras is central to cellular responses of lymphocytes, and is becoming more complex as new information is acquired. However, the precise role of this protein in cellular responses mediated by NK cells in response to IL-2 or to sensitive targets is not known.

HYPOTHESIS:

The **hypothesis** was that the downstream events which are generated as a consequence of Ras activation are sufficient, and may be necessary, for processes leading to NK cell-mediated cytolysis.

OVERALL OBJECTIVE:

NK cell function is a result of a sensitive balance between inhibitory and activation signals, mediated by a variety of cellular receptors. In other systems, and to some extent in NK cells, activating receptors such as CD28, CD45, CD16, integrins, and cytokine receptors have been linked to Ras activation, as well as other signalling proteins. How the activation of this protein affects the cascade of events involved in NK function and leading to cytolysis is not clear. This proposal's main objective was to address the question of the intracellular changes dependent on the activation of the Ras pathway, and the latter's involvement in the multiple facets of NK function.

MATERIALS AND METHODS

Maintenance of Cell Lines

The NK3.3 cell line was obtained from Dr. J. Kornbluth (University of Arkansas) (Kornbluth *et al.*, 1982). This cell line is recognized by the scientific community as a true human NK cell clone. The NK3.3 clone was maintained in RPMI-1640 containing 15% fetal calf serum (FCS) (Gibco-BRL, Burlington, ON), supplemented with 100 U/ml recombinant IL-2 (NCI, Biological Response Modifiers Program, Frederick, MD). Cultures were set up at $2x10^5$ cells/ml in 25 cm^2 flasks (Nunc, Gibco-BRL, Burlington, ON), in a volume of 15 ml. Cells were incubated in a humidified incubator, at 37° C and 5% CO_2, and fed every 3-4 days with fresh medium.

The K562 cell line and the Raji cell line were used as target cells in the cytotoxicity assays. K562 is a NK-sensitive, LAK-sensitive human erythroleukaemia cell line (Lozzio and Lozzio, 1975). Raji is a NK-resistant, LAK-sensitive cell line derived from a Burkitt's lymphoma (Pulvertaft, 1964). Both cell lines were obtained from the American Type Culture Collection (ATCC) (Rockville, MD). K562 and Raji were maintained in RPMI-1640 with 10% FCS at 37° and 5% CO_2.

The Daudi cell line is a B cell lymphoma, obtained from ATCC, that was used as feeder layer for the cloning of the NK3.3 cell line. Daudi cells were maintained in RPMI-1640 with 15% FCS, at 37° C and 5% CO_2.

The PG13 cell line is a packaging line containing the gibbon ape leukaemia virus (GaLV) envelope and capable of producing retrovirus vectors capable of infecting human cells (ATCC)(Miller *et al.*, 1991). This cell line was transfected with the constructs MSCV-v-H-ras and MSCV-neo for the creation of the cell lines PG13-MSCV-ras and PG13-MSCV- neo, which were gifts from Dr. J. E. Dick. The MSCV constructs were a gift from Dr. R.G. Hawley and previously described (Hawley *et al.*, 1995). In these retroviral vectors the ras oncogene is driven by the powerful promoter of the long

terminal repeats (LTR) of the murine stem cell virus, and the neomycin resistance is controlled by the phosphoglycerate kinase (PGK) promoter The PG13-MSCV-neo was maintained in our laboratory in DMEM with 10% FCS. The PG13-MSCV-ras was maintained in a Level 3 laboratory in the same medium.

HeLa cells are a cervical adenocarcinoma cell line (ATCC).

2H1 is a cell line derived from C3H $10T^{1/2}$ murine embryonic fibroblast cell line transfected with a pSV_2-neo plasmid containing the coding sequence of ras^{Val-12} under the control of the mouse metallothionein-I promoter.

Mononuclear Cell Preparation

Peripheral blood was collected by venipuncture from healthy adult donors into heparinized Vacutainer tubes (Becton Dickinson Canada Inc., Mississauga, ON). Peripheral blood mononuclear cells were prepared by centrifugation over Ficoll-Isopaque (SG 1.077 g/ml) (Pharmacia, Baie d'Urfe, Quebec)(Boyum, 1968).

Preparation of Electro-Competent Bacteria

Electro-competent bacteria were prepared as follows (Sambrook *et al.*, 1989b): 10 ml of Luria Bertani (LB) broth were inoculated with a single colony of *E. coli* DH5α grown overnight and added to a liter of LB. Then, the bacteria were grown at 37° with vigorous shaking to an OD_{600} of 0.5 – 1. The flask was then put on ice for 15 – 30 minutes, and the cells were pelleted by centrifugation at 4000xg for 15 min. The medium was removed and the cells were resuspended in 1 liter of cold sterile H_2O. The cells were pelleted again and resuspended in 0.5 liter of water, followed by a wash in 20 ml cold 10% glycerol. The cells were pelleted by centrifugation and resuspended in 2 –3 ml of cold 10% glycerol. Finally, the cells were aliquoted in 40 µl aliquots and frozen at -70°C for further use.

Electro-Transformation

The electro-competent DH5α cells (40 µl) were thawed gently on ice and mixed with 1 –2 µl of the DNA preparation. The mixture was transferred to an electroporation cuvette (Bio-Rad Laboratories, CA) at 4° C and the electroporation was performed in a Gene Pulser apparatus (Bio-Rad Laboratories) set at 25 µF capacitance and 2.5 kV. The pulse controller was set at 200 Ohms. The bacteria were allowed to recover in 1 ml of SOC media (100ml:2 g tryptone, 0.5 g yeast extract, 0.06 g NaCl, 0.36g dextrose, 0.02 g $MgCl_2$ and 0.25 g $MgSO_4$), and plated on LB plates containing the appropriate selective antibiotic.

Plasmid Extractions

For small scale plasmid preparations an adaptation of the method described by Sambrook *et al.* (1989a) was used. A single colony of the bacteria was inoculated in 5 ml of Terrific Broth (TB) containing 100µg/ml ampicillin. The tubes were incubated with shaking at 37° C for 16h. 1.5 ml of the culture were transferred into microcentrifuge tubes, and centrifuged at 12,000 rpm for 2 min. The pellet was resuspended in 100 µl of 50mM glucose, 25 mM TrisHCl pH 8.0 and 10 mM EDTA pH 8.0 at 4° C. Then 200 µl of freshly prepared 0.2 N NaOH and 1% SDS were added to the tubes, mixed by inversion and incubated on ice for 10 min. 150 µl of a solution of 60 ml 5M potassium acetate, 11.5 ml glacial acetic acid and 28.5 ml H_2O was added and mixed, and the mixture was centrifuged at 12,000 rpm for 15 min. The supernatant was treated with RNase (Sigma Chemical Co., Oakville, ON) for 1 hour at 37° C. An equal volume of 1:1 phenol-chloroform was added and mixed, followed by centrifugation at 15,000 rpm for 15 min. The DNA in the aqueous fraction was then precipitated with 2 volumes of 100% ethanol (15,000 rpm for 20 min), and the pellet was washed two times with 70% ethanol. The ethanol was removed and the DNA was air-dried and resuspended in sterile H_2O.

Large Scale Preparation (Midi-Prep) of Plasmid DNA

Plasmid DNA was prepared using QIAGEN plasmid purification kits (QIAGEN Inc., CA) following the manufacturer's instructions. 25 ml of LB with 100 μg/ml ampicillin were inoculated with a colony of bacteria containing the plasmid of interest, and incubated overnight at 37° C with vigorous shaking. The bacteria were harvested by centrifugation at 4° C for 15 min at 6,000 x g and resuspended in 4 ml of suspension buffer (50 mM Tris-HCl, 10 mM EDTA pH 8.0, 100 μg/ml RNase A) to which 4 ml of lysis buffer were added (0.2 M NaOH, 1% SDS). The mixture was incubated at room temperature for 5 min and 4 ml of neutralization buffer (3.0 M CH_3COOHK, pH 5.5) were added. After an incubation on ice for 20 min the mixture was filtered through sterilized cheese cloth and applied onto an equilibrated QIAGEN tip-100 washed with 4 ml of equilibration buffer (750 mM NaCl, 50 mM MOPS, 15% ethanol, 0.15% Triton X-100, pH 7.0) and allowed to enter the resin by gravity flow. The QIAGEN tip was washed with 2x10 ml of wash buffer (1.0 M NaCl, 50 mM Tris-HCl, 15% ethanol, pH 7.0), and the DNA was eluted from the column with 5 ml elution buffer (1.25M NaCl, 50 mM Tris-HCl, 15% ethanol pH 8.5). The DNA was precipitated with 3.5 ml isopropanol, and centrifuged at 15,000 x g at 4° C for 30 min. The DNA pellet was washed with 2 ml 70% ethanol and air-dried. The DNA was resuspended in 100 μl of TE (10 mM Tris-HCl, pH 8.0, 1mM EDTA). The yield was quantitated by measuring the optical density at 260 nm and calculated by the equation: DNA concentration (μg/ml) = dilution fold x 50 x OD_{260}.

Agarose Gel Electrophoresis

Agarose gels were prepared by dissolving 0.8% (w/v) agarose (ICN Biomedicals Inc., Montreal, PQ) in TAE buffer (40 mM Tris, 0.6% glacial acetic acid, 5mM EDTA, pH 8.0) with 1 μg/ml ethidium bromide (Sambrook *et al.*, 1989). DNA samples were loaded in the presence of loading buffer (5% glycerol, 0.04% xylene cyanol FF and

0.04% bromphenol blue) and resolved by electrophoresis in TAE buffer at constant voltage. DNA fragments were visualised under ultraviolet light, and sized by comparison to 1 Kb DNA ladder (GIBCO-BRL, Burlington, ON) or the 7DNA/*Eco*I301/*Mlu*1 ladder (MBI Fermentas, Hamilton, ON).

Purification of DNA Fragments

When a specific band of DNA was needed the DNA was excised from the gel by one of the two methods described below. First, the DNA was isolated from a low melt agarose (ICN Biomedicals Inc., Montreal, PQ) gel with a razor blade and applied onto a glass wool column (Sambrook *et al.*, 1989a). The column was centrifuged at 400 x g for 2.5 min, and the DNA was precipitated from the solution with 1 µl of glycogen (20 mg/ml) (Boheringer Manheim, Laval, PQ), 0.1 vol. of 4 M LiCl and 3 vol. of cold 100% ethanol by centrifugation at 4° C at 11,000 x g for 20 min. The second method used was using Prep-A-Gene (Bio-Rad, Laboratories, CA), according to the manufacturer's instructions. The band of interest was excised from a regular agarose gel and placed in a microcentrifuge tube in the presence of three volumes of 6 M sodium perchlorate (Bio-Rad Laboratories, CA). The agarose was melted at 55° C and 5 µl of the Bio-Rad Prep-A-gene matrix per 1 µg of DNA were added. The matrix was washed with 200 µl of washing buffer (50% ethanol, 0.8 M NaCl, 40 mM Tris, 4 mM EDTA, pH 7.5) and centrifuged for 5 seconds at 12,000 rpm. The DNA was eluted with 20 µl of TE buffer at 55° C for 5 min.

Preparation of DNA for Transfections

Restriction endonucleases and DNA modifying enzymes were purchased from GIBCO-BRL, or New England Biolabs (New England Biolabs, Ltd., ON). Restriction digestion reactions were performed according to the manufacturer's instructions. The plasmids p-MT-H-ras and the plasmid PGK-neo-pGEM7 used for the transfection

experiments were gifts from Dr. G. Filmus (University of Toronto) and Dr. J. Rossant (University of Toronto) respectively. For transfections, the p-MT-H-ras was propagated in *E. coli* isolated by QIAGEN and linearized by digestion with *Sca*I. The plasmid PGK-neo-pGEM7 was prepared in the same fashion but digested with *Xba*I. After the digestion the linearised DNA was visualized on a 0.8% agarose gel to ensure digestion. The DNA was cleaned by the addition of 1 volume of 1:1 phenol chloroform. The aqueous fraction was transferred to a clean microcentrifuge tube and the DNA was precipitated from the solution with 50 µl of 3M potassium acetate and 2.5 volumes of 100% ethanol. The DNA was centrifuged at 13,000 rpm for 30 min at 4° C, washed with 500 µl of 70% ethanol and air-dried for 10 min. The DNA was resuspended in 100 µl of TE, and passed through a 0.22 micron filter.

Transfection of the NK3.3 Clone with MT-Ras^{Leu-61}

Two x 10^5 NK3.3 cells were seeded in RPMI-1640 with 15% FCS and 100 U/ml IL-2 in 75 cm^2 flasks in a volume of 50 ml. On the fifth day the cells were harvested into a 50 ml tube. 4x10^7 cells were resuspended in 0.8 ml in ice cold PBS (without Ca^{++} and Mg^{++}) containing 40 µg of the linearised MT-Ras^{Leu-61} plasmid and 10 µg of the linearised resistance PGK-neo plasmid. The cells were transferred to a 0.4 mm electroporation cuvette (Bio-Rad Laboratories, CA) and the electroporation was performed using a gene pulser electroporator (Bio-Rad Laboratories, CA) at 250 Volts and 960µF capacitance (Potter, 1992). The cells were then put on ice for 20 min, resuspended in culture media and allowed to recover for 48h before the selection media was applied. For selection of transfectants geneticin (G418) (GIBCO-BRL, Burlington, ON) at 500 µg/ml were added to the culture medium.

NK3.3 Cloning

NK3.3 cells selected in G418 containing media were cloned by limiting dilution. The cells were plated at a density of 0.36 cells/well in 100 µl RPMI-1640 with 15% FCS, 100 U/ml IL-2 and 500 µg/ml G418 in a 96-well flat-bottomed microtitre plate. Daudi cells were irradiated at 10,000 cGy using a Caesium (^{137}Cs) irradiator, and were added as a feeder layer at $1x10^5$ cells/well. The cells were grown until colonies were apparent expanded to 24 well plates (Nunc, Gibco-BRL, Burlington, ON) and grown for further study, or frozen in liquid nitrogen.

NK3.3 Infection with MSCV-v-Ras

Three flasks containing 2×10^5 NK3.3 cells and 80% confluent packaging lines PG13-MSCV-v-H-Ras or with PG13-neo were set up. Two of the flasks contained RPMI-1640, 15% FCS and 100 U/ml IL-2 and one flask was maintained in the same medium without IL-2. The cells were co-cultured for 72 h in a Level 3 laboratory (Hospital for Sick Children, Toronto, ON.). The PG13 cell line is a packaging line harboring high titres of a retrovirus derived from the gibbon ape leukaemia virus and containing v-H-ras oncogene with arginine at position 12. Pre-infection, the PG13 cell lines were irradiated at 6,000 cGy. After the infection the NK3.3 cells were transferred to a new flask, and allowed to recover for 24 h in culture medium with or without IL-2. Then, the cells were subjected to selection medium (RPMI-1640, 15% FCS, 100 U/ml IL-2, 1 mg/ml G418) or to the same medium in the absence of IL-2 for the third flask.

Detection of Helper Virus

In order to be able to transfer the cells to a Level 2 facility, the cells had to be tested for the presence of helper virus, to ensure that the infected NK3.3 cells were unable to generate infectious particles (Cepko, 1996). For this purpose, the infected NK3.3 cells were grown to sub-confluency after which the supernatant was removed.

One mililitre of the supernatant was filtered through a 45 μm filter, 8 μg/ml polybrene (Sigma Chemical Co., Oakville, ON) was added, and applied onto a 60 mm tissue culture dish (Nunc, Gibco-BRL, Burlington, ON) containing a 1:50 dilution from a sub-confluent culture of HeLa cells. The cells were incubated at 37° C, 5% CO_2 for 2 h, and 3 ml of culture medium (DMEM, 10% FCS) were added. The cells were then grown to confluence and split once more 1:50 into fresh dishes. 2 μg/ml polybrene were added, and the cells were grown to 50% confluence. At this point the medium was discarded, 4 ml of fresh culture medium were added, and the cells were grown to confluence. The supernatant was collected, filtered through a 0.45 μm filter, and 8 μg/ml polybrene were added. One ml of the supernatant was added to 60 mm tissue culture dishes containing 2 x 10^5/dish HeLa cells. The cells were incubated for 2 h at 37° C, 5% CO_2 and 2 ml of fresh culture medium were added. The cells were then transferred to a 100 mm culture dish and selective medium (DMEM, 10% FCS, 400 μg/ml G418) was added. As a control, HeLa cells that were grown in culture medium all along were set up. The selective medium was changed every 3 days until the control cells had died, and the presence of G418 resistant colonies had been tested.

Preparation of Cell Lysates

NK3.3 sublines and clones were grown to confluence in 75 cm^2 flasks. 0.2 - 1 x 10^8 cells were harvested into 50 ml polyethylene tissue culture tubes (Nunc, Gibco-BRL, Burlington, ON), washed with cold PBS and lysed in 1 ml of lysis buffer (50 mM Tris-HCl pH 7.5, 100 mM NaCl, 5 mM $MgCl_2$, 1% Triton X-100, 1 mM PMSF, 10 mM leupeptin, 10 μg/ml aprotinin) at 4° C for 20 min. The lysates were transferred to microfuge tubes and precleared by centrifugation for 10 min at 10,000 rpm.

SDS-Polyacrylamide Gel Electrophoresis (PAGE)

Protein concentration was determined by Pierce BCA reagent (Pierce, Rockford, IL). Ten micrograms of protein were boiled in water at 100° C in 1 x sample buffer (62.8 mM Tris-HCl pH 6.8, 10% glycerol, 2% SDS, 0.01% phenol red, 0.04 M DTT) prior to loading on a discontinuous SDS-PAGE consisting of a 12 % resolving gel and 3% stacking gel according to the method described by Laemmli (1970). Gels were prepared using a Bio-Rad minigel apparatus and electrophoresed at 125 V for 2 h.

Immunoprecipitation

Two x 10^7 cells were lysed in 1 ml lysis buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% NP 40 Nonidet-P 40, 1% Sodium Deoxicholate, 0.1% SDS, 1 mM PMSF, 10 μg/ml aprotinin, 10 μg/ml leupeptin) for 20 min at 4° C. Debris was discarded from the lysates after centrifugation at 13,000 rpm for 15 min at 4° C. The supernatants were precleared by incubation, with gentle mixing, using 30 μl of goat anti-rat IgG Sepharose (Sigma, Mississauga, ON) for 1 h at 4° C, followed by pulse centrifugation. To the supernatants 50 μl of goat anti-rat IgG sepharose and 3 μg of the Y13-259 anti-ras antibody (Oncogene Research Products, Cambridge, MA) were added. Ras was immunoprecipitated overnight at 4° C with gentle mixing. The mixture was then washed four times with cold PBST, and the supernatant was discarded. The sepharose was then resuspended in 20 μl of SDS sample buffer, boiled for 5 min and centrifuged for 2 min prior to SDS-PAGE analysis.

Western Blotting

Proteins separated by SDS-PAGE were transferred to nitrocellulose (Hy-Bond, Amersham ECL, Oakville, ON) by electrical current for 1 h at 100 V in transfer buffer (0.025 Tris, 0.192 glycine, 0.1% SDS, and 10% methanol)(Gallagher *et al.*, 1993). Membranes were blocked for 1 h with PBS containing 10% skim milk powder, and washed with PBST (PBS with 0.5% Tween-20). The membranes were then incubated

overnight with the primary antibodies, (pan-ras Ab 1-3, or pan Ras^{Arg-12}, Oncogene Research Products, Cambridge, MA) at a concentration of 5-10 μg/ml in PBST 1% skim milk powder at 4° C. After washing three times in PBST, the membranes were incubated for 2 h at room temperature, with goat anti-mouse HRP-conjugated antibody (Jackson Laboratories, Jackson Immunoresearch Products, Bar Harbor, ME) at 1:20,000 dilution in PBST with 1% skim milk powder. After rinsing three times in PBST, membranes were developed using an ECL reagent kit (LumiGlo, Kirkegaard and Perry Laboratories, Inc., Gaithersburg, MD., or Amersham, Oakville, ON).

RNA Extraction

RNA was isolated using TRIzol (Gibco-BRL, Burlington, ON) (Gilman and Sacchi, 1992). One x 10^7 cells were pelleted by centrifugation and lysed in 1 ml TRIzol reagent according to the manufacturer's instructions. The homogenised samples were transferred to a microcentrifuge tube, and incubated for 5 min at room temperature. 0.2 ml chloroform were added, the tubes were shaken vigorously and incubated at room temperature for 2 min. Then, the samples were centrifuged for 15 min at 4° C at 12,000 x g. The aqueous phase containing the RNA was transferred to a new tube and the RNA was precipitated with 0.5 ml isopropanol. After incubation at room temperature for 10 min, the samples were centrifuged at 12,000 x g for 15 min. The supernatant was removed and the RNA was washed with 75% ethanol. After the pellet was air dried, the RNA was resuspended in RNase-free water and frozen at – 80°C for 24 - 48 h. The OD_{260} of each sample was read in a spectrophotometer. The amount of RNA was determined by the equation: RNA concentration (μg/ml) = dilution fold x 40 x $O.D._{260}$. All glassware plasticware and solutions were RNase-free.

Northern Blotting

Northern blotting was done according to Brown (1993). Ten μg total RNA were adjusted to a total of 5 μl volume with DEPC (diethylpyro-carbonate)-treated autoclaved water and 25 μl of sample buffer (0.75ml formamide (Fisher, Chemicals) 0.15 ml 10 x MOPS/EDTA buffer (0.2 M MOPS 3-(N-morpholino) propanesulfonic acid (Fisher), 50 mM sodium acetate, 10 mM EDTA, pH 7.0, 0.24 ml formaldehyde, 0.1 ml RNase free water, 0.1 ml glycerol, 10% bromophenol blue) were added. The samples were heated at 65° C for 15 min prior to loading on an agarose formaldehyde gel (2.1 g agarose dissolved in 152.2 ml H_2O and cooled to 50° C, 5.25 ml 37% formaldehyde (0.66M), 17.5 ml of 10 x MOPS/EDTA-buffer). The gel was run at 30V for 18 h or at 85v for 3- 5 h, in electrophoresis buffer (1 x MOPS/EDTA). The gel was soaked for two 20 min periods in 10 x SSC (1.5 M NaCl, 0.15 M Na_3 citrate·H_2O, pH 7.0) with gentle shaking, and transferred to a positively-charged nylon membrane (Amersham, Oakville, ON) pre-wetted 5 min in H_2O and 5 min in 10 x SSC, by capillary action. The RNA was fixed on the membrane by baking at 80° C for 2 h in a vacuum oven. The membrane was placed in a hybridization bag in prehybridization solution (50% formamide, 5 x Denhart's solution (1% Ficoll (Pharmacia, Uppsala), 1% BSA (Sigma-Aldrich Canada,Oakville, ON), 1% polyvinylpyrrolidone (Sigma), 5 x SSPE (1.5M NaCl, 0.1 M NaH_2PO_4·H_2O, 0.01M EDTA, pH 7.4), 200 μg/ml herring sperm DNA (Sigma) denatured in 0.2 M NaOH for 5 min, and neutralized with 0.2 M Acetic acid, for 4 h at 42° C, in a water bath with gentle shaking.

Preparation and Labeling of Probes

The DNA probe for v-H-Ras was amplified from the MSCV-v-H-Ras plasmid (Dr. R. Hawley, University of Toronto) by polymerase chain reaction (PCR)(Kramer and Coen, 1997). A 500 bp fragment, specific for v-H-ras, was amplified from the plasmid using the primers 5' AAGAGTGCCCTGACCATCC 3' and 5'

GACAGCACACAGTTGCAGC 3' synthesized at Cortec, DNA Laboratories Inc., Dept. of Biochemistry, Queen's University. The PCR reaction containing 2 units of Vent polymerase (New England Biolabs,Mississuaga, ON) in 100 µl mixture of 1 x supplied buffer, 200 µM dNTPs, 1 µM of each primer and 500 ng template DNA was run in a Perkin Elmer GeneAmp PCR System 2400. DNA was first denatured at 94° C for 2 min prior to the addition of the Vent polymerase. Then, the reaction was run for 25 cycles of 1 min at 94° C, 1.30 min at 48° C (T_m of the primers), and 1.30 min at 74° C. An additional 3 cycles of 5 min each at 74° C and 37° C, to allow for elongation and repair, were run before the reaction was stopped. The GAPDH probe of 780 bp was released from the plasmid pHcGAP (ATCC) by digestion with *Pst* I and *Xba* I (New England BioLabs). Both fragments from the PCR reaction or the digested plasmid were run on an agarose gel, isolated as described above, and used as probes.

The probes were labelled by random oligonucleotide-primed synthesis (Tabor, 1991). 50 ng of the DNA fragments were diluted in 32 µl of H_2O, denatured by boiling for 3-4 min and chilled on ice. Then the probes were mixed with 10 µl of labeling buffer (100 µM dCTP, 100 µM dGTP, 100 µM dTTP (Pharmacia Biotech, Baie d'Urfe, PQ), 50 mM Tris-HCl pH 7.5, 50 mM Mg_2Cl_2, 250 mM NaCl, 50 units of pd $(N)_6$ (Pharmacia Biotech), 25 mM DTT, 50 µCi of [α-^{32}P]dATP (ICN Biomedicals Inc.), and 2 units of Klenow fragment (New England Biolabs, Mississauga, ON)). The reaction was incubated for 1.5 h at room temperature. The unincorporated ^{32}P-dATP was removed by passing the mixture twice through Sephadex G-50 (Pharmacia Biotech) columns. A specific activity of 1 x 10^8 cpm/µg DNA was achieved.

The labeled probes specific for v-H-ras and GAPDH were added to the hybridization bag containing the membrane and incubated for 18 h at 42° C. The membrane was washed three times in 0.1%SDS/0.1 x SSC prior to autoradiography.

Cytotoxicity Assay

NK activity was measured in vitro using an 18 hour ^{51}Cr release assay against the targets K562 and Raji, as described previously (Pross *et al.*, 1981). Target cells were labeled with 100 μCi of $Na_2{}^{51}CrO4$ (NEN, Boston, MA) for 1 h at 37° C, washed three times and resuspended at a concentration of 5 X 10^4 cells/ml. One hundred microlitres of ^{51}Cr-labelled targets were added to a 96-well V-bottom microtiter plate (Corning Costar Corporation, Cambridge, MA) which had been previously loaded with 50 μl of serially diluted effector cells, or medium. The ratios used were routinely 4 serial dilutions starting at an effector to target (E/T) ratio of 20:1. The cells were incubated for 18 h at 37° C, 5% CO_2, in a humidified incubator. The plates were then spun for 10 min at 200 x g and 100 μl of the supernatant were removed from each well. The supernatant was counted for radioactivity in a Beckman 5500 gamma counter. Specific ^{51}Cr release was assessed as follows:

$$\% \text{ Specific Release} = \frac{\text{cpm (test)} - \text{cpm (spontaneous release)}}{\text{cpm (max)} - \text{cpm (spontaneous release)}} \times 100$$

Proliferation Assay

NK3.3 cell lines were washed and resuspended in media without IL-2 and cultured overnight at 37° C 5% CO_2. Then the cells were resuspended at 0.5 X 10^6 cells/ml. The proliferative response was measured by culturing 5 x 10^4 cells /well in a 96-well flat-bottomed microtitre plate (Nunc, Gibco-BRL, Burlington, ON) with and without various cytokines for 48 hours. Tritiated thymidine ([^{3}H]TdR, 1 μCi/well) (New England Nuclear, Boston, MA) was added 18 hours before harvesting with an automated multiwell Skatron Titertek cell harvester (Skatron Instruments, Inc. Sterling, VA) that aspirates cells, lyses cells, and transfers DNA onto filter paper while allowing unincorporated [^{3}H]TdR to wash out. Counts per minute were measured using a Beckman LS 6000LL scintillation counter (Garvey *et al.*, 1977).

Cytokine Production

Cytokine production was measured using ELISA kits (ID Labs Biotechnology, London, ON), according to the manufacturer's instructions. Cells were set at 1 $x10^6$ cells/ml in RPMI 10% FCS in 24 well plates (Nunc, Gibco-BRL) in the presence or absence of 500 U/ml IL-2 for 24 h or 72 h. Then, the cells were centrifuged for 500 x g for 10 min, and 100 µl of the supernatants were added onto microtitre plates pre-coated rat anti-human IFNγ, TNFα or IL-2. In addition supplied standards were used. Rabbit anti-human IFNγ, TNFα or IL-2 polyclonal antibodies were added and the plates were incubated for 3 hours at room temperature. The plates were washed and a goat anti-rabbit alkaline phosphatase-conjugated antibody was added. The plates were incubated for 45 min at room temperature, and washed prior to the addition of the substrate. The colourimetric reaction was assessed at 492 nm using an ELISA plate reader (Titertek).

Granule Exocytosis

Total granule content and percent granule exocytosis were measured according to the method of Taffs and Sitkovsky (1993). NK3.3 sublines were set up at $5x10^5$ cells/well in 50 µl of RPMI with 10% FCS in a 96 well flat-bottomed microtitre plate (Nunc, Gibco-BRL, Burlington, ON). One x 10^6 cells/50 µl of culture medium or K562 were added to the plate. For the measurement of spontaneous granule exocytosis the cells were incubated with medium alone. The cells were then incubated for 3 – 4 h at 37° C, 5% CO_2 in a humidified environment. After the incubation the plates were centrifuged at 500 x g for 10 min and 40 µl of the supernatants were collected and assayed for serine esterase activity by addition of 180 µl of the reaction mixture - PBS (phosphate buffer saline) pH 7.0 with 2 X 10^{-4} M N-benzylocarboxy-L-lysine thiobenzil ester (BLT) (Calbiochem, San Diego, Ca), and 1.1 X 10^{-4} M 5.5-dithiobis (2-nitrobenzoic acid) (Sigma Chemicals Co., Oakville, ON). For intracellular serine esterase activity 1 x 10^6

cells were lysed in PBS containing 0.5% NP-40. 40 µl of the lysates were mixed with 180 µl of the reaction mixture. The reaction was allowed to proceed for 30 min at room temperature before the plates were read at 412 nm wavelength in an ELISA plate reader (Titertek). The percentage granule exocytosis in response to K562 was calculated as follows:

$$\% \text{ Granule Exocytosis} = \frac{O.D._{.412}\,(K562) - O.D._{.412}\,(\text{medium})}{O.D._{.412}\,(\text{intracellular}) - O.D._{.412}\,(\text{medium})} \times 100$$

Conjugate Formation Assay

Conjugates were enumerated by fluorimetry according to Lebow *et al.* (1986). NK3.3 cell lines and K562 target cells were washed in PBS at 4° C prior to adjusting the cells to 1 x 10^6 cells/ml in 1% BSA in PBS. The effectors were stained with calcein acetoxymethylester (calcein AM, Molecular Probes, Eugene OR), at 0.5 µg/ml and the targets were stained with hydroethidine (Molecular Probes) at 60 µg/ml, for 1 h at room temperature in the dark. The cells were then washed in ice-cold PBS and resuspended in PBS with 5 mM $MgCl_2$, and 1 mM ethylene glycol-bis (β-aminoethylether)-N,N',N'-tetraacetic acid (EGTA). Conjugates were formed by mixing 2 x 10^5 effector cells with the same number of target cells in 5 ml flow cytometry tubes. The cells were pelleted by centrifugation at 180 x g for 1 min, and incubated for 30 min at 37° C. The tubes were placed on ice, and the pellets were gently resuspended before analysis on a Coulter EPICS Elite flow cytometer (Coulter Electronics of Canada, Burlington ON). All double positive events were enumerated as conjugates.

Flow Cytometry

Flow cytometric analysis was performed using a Coulter EPICS Elite flow cytometer. Cells were washed in RPMI without serum and pelleted by centrifugation. An

aliquot of 0.5 – 1 x 10^6 cells were directly labeled by incubating with fluorescein or phycoerythrin-conjugated antibodies (Immunotech, Division of Coulter, Burlington, ON., Sigma Immunochemicals, Oakville, ON.) for 45 min on ice. The cells were then washed twice in RPMI-1640, 1% BSA, and resuspended in 500 µl RPMI-1640 with 1% paraformaldehyde prior to analysis.

Data Processing

All ^{51}Cr release data were expressed in lytic units, defined as the number of effectors required to lyse 20% of the target cells per million (LU (20%)/10^6), calculated according to the equation $y=A(1-e^{-kx})$, where y= fraction ^{51}Cr release, x= lymphocyte: target cell ratio, k= a constant proportional to effector cell number and equal to the negative slope of the target cell survival curve obtained by plotting ln(A-y) vs. x, and A= the maximal cell mediated lysis (Pross *et al.*, 1981).

Means, standard errors, standard deviations and p values were determined using the Student's paired and unpaired 't' and calculated using Slidewrite Plus for Windows (4.1). Analysis of variance (ANOVA) and correlation coefficients were performed using Microstat II.

RESULTS

The main objective of this project was to create stable NK3.3-derived cell lines expressing the activated form of $p21^{ras}$. The experiments described in this section document the production of these cell lines, and their characterization with respect to Ras activation and its effect on cytotoxic function.

I. Inhibition of Ras Function and Expression of Constitutively Activated Ras in NK3.3

NK3.3 - The Cell Line Used in the Model System

The NK3.3 clone was established by Dr. J. Kornbluth (University of Arkansas) and has been described in the introduction. A characteristic of these cells is that they gradually lose their cytotoxic ability, with time in culture. Typical NK3.3 cytotoxicity data over a period of 9 months in culture are shown in Figure 1 (percent ^{51}Cr release) and Figure 2 (lytic units). These data were taken from experiments using cells derived from the original "fresh thawed" cells after uninterrupted culture for the times indicated. When first tested, the NK3.3 cells were highly cytotoxic. After a period of 3 months their activity was about 25% of the starting level, and by 9 months the cells had lost their cytotoxic capability. There was no loss of viability of the NK3.3 cells over this time period. Controls done at each of these time points (data not shown) also indicated that there had been no change in target sensitivity, or other characteristics of the assay system over the course of the experiments. This characteristic of the cell line enabled us to study the effect of expression of constitutively activated $p21^{ras}$ on NK 3.3 function at different levels of cytotoxic capability.

Figure 1. Cytotoxicity of the NK3.3 clone in culture. NK3.3 cells were maintained in culture for at least nine months, and the cytotoxicity of the clone was measured at several time intervals. NK3.3 cells were stimulated with 100 U/ml IL-2 prior to analysis for cytolytic activity in an 18h ^{51}Cr release assay against the NK-sensitive target K562, as described in Materials and Methods. Cytotoxicity is expressed as percent ^{51}Cr release.

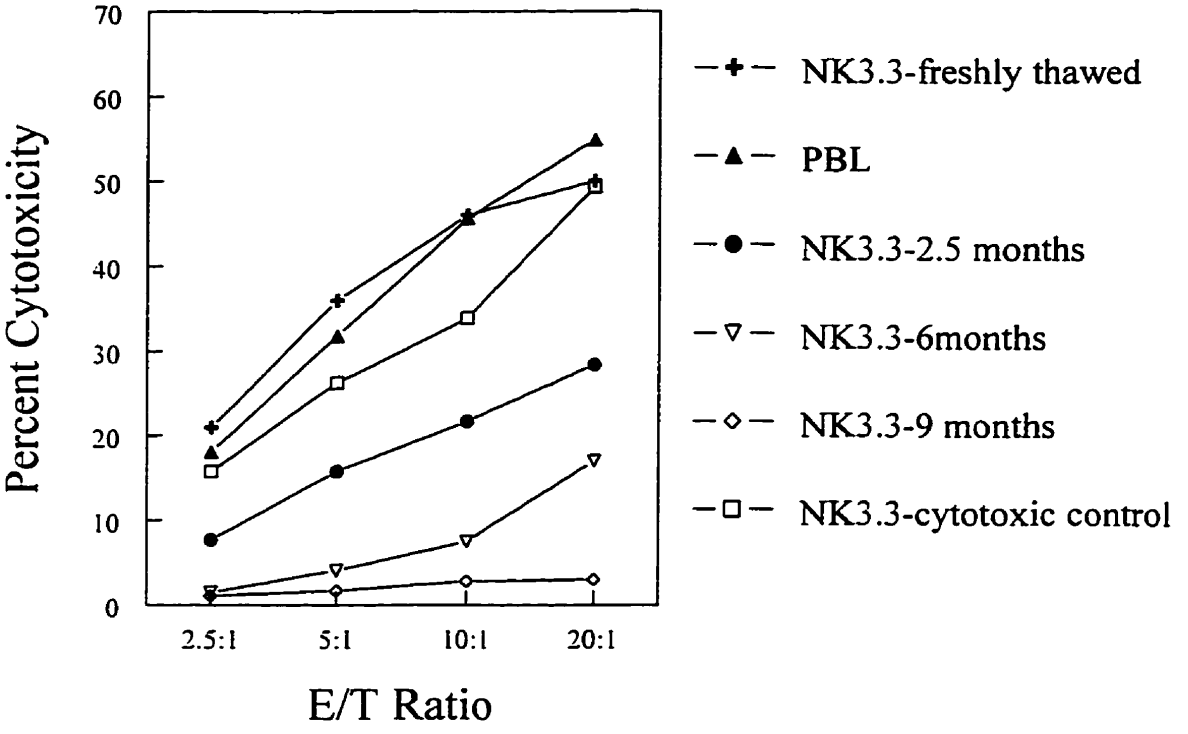
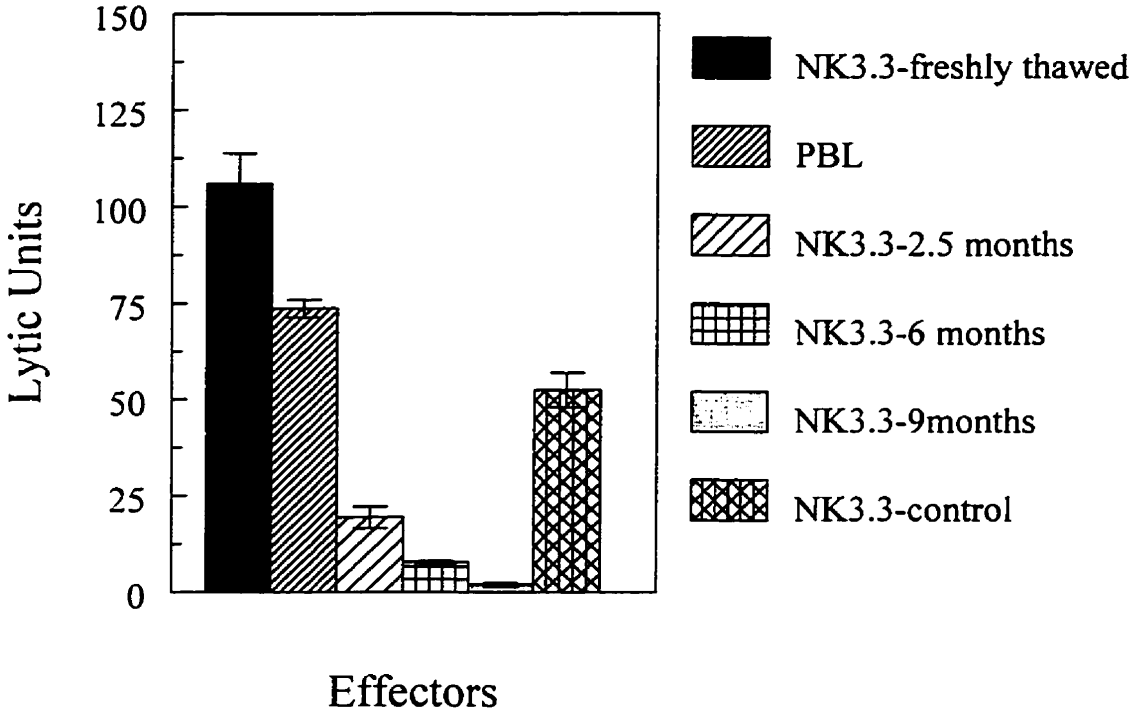

70
60
50
40
30
20
10
0
Percent Cytotoxicity
2.5:1
5:1
10:1
20:1
E/T Ratio
NK3.3-freshly thawed
PBL
NK3.3-2.5 months
NK3.3-6months
NK3.3-9 months
NK3.3-cytotoxic control

Figure 2. Cytotoxicity of the NK3.3 clone in culture. NK3.3 cells were maintained in culture for at least nine months, and the cytotoxicity of the clone was measured at several time intervals. NK3.3 cells were stimulated with 100 U/ml IL-2 prior to analysis for cytolytic activity in an 18h ^{51}Cr release assay against the NK-sensitive target K562, as described in Materials and Methods. Cytotoxicity of the NK3.3 clone expressed in lytic units (20%)/10^6 cells +/- S.D. The long-term cultures are significantly different from controls and from each other ($p<0.05$).


150
125
100
75
50
25
0
Lytic Units
Effectors
NK3.3-freshly thawed
PBL
NK3.3-2.5 months
NK3.3-6 months
NK3.3-9months
NK3.3-control

The Effect of the Farnesyl Inhibitor B956 on Natural Killer Cell Cytotoxicity

$p21^{ras}$ is one of the few proteins which undergoes farnesylation as a post-translational change (Lobell, 1998). After translation, a farnesyl group is added to the carboxy-terminus of the protein mediated by the enzyme farnesyl transferase. This change enables the protein to anchor at the cellular membrane, and to transmit intracellular signals from extracellular receptors, such as growth factor and cytokine receptors (Sun *et al.*, 1998). Other proteins which undergo similar postranslational changes are the family of Rho proteins (Lobell, 1998). Therefore, inhibition of farnesylation is not entirely Ras specific. To provide us with information on the possible involvement of Ras in NK cell cytotoxicity we investigated the effect of the farnesyl transferase inhibitor, B956 on the cytolytic activity of NK cells as measured against the NK-sensitive target K562. Figures 3 and 4 describe the inhibition of NK3.3 cytotoxicity by the compound B956 expressed as percent cytotoxicity (Fig 3) and as lytic units (Fig 4). NK3.3 cells were treated with various concentrations of B956. Because B956 has to be reconstituted in DMSO, corresponding dilution of DMSO were used as controls. The cells were then tested in an 18h ^{51}Chromium release assay against the NK-sensitive cell line K562. The data show that B956 inhibited cytotoxicity at all concentrations tested, with a maximal inhibition at 150 μM. Inhibition was significant ($p<0.05$) at concentrations of 100 μM and150 μM B956, compared with the untreated control cells and the corresponding dilutions of DMSO. However, DMSO itself has an inhibitory effect on NK3.3-mediated cytotoxicity at the dilutions corresponding to 100 and 150 μM B956. Therefore, at high concentrations of the diluent, B956 appears to have the same effect as at the intermediate concentration.

Figure 3. Effect of the farnesyl transferase inhibitor B956 on NK3.3 cytotoxicity (Chromium release). NK3.3 cells were treated with B956 at the concentrations indicated or with DMSO as control, for 48h prior to assessment of cytotoxicity in a ^{51}Cr release assay in the presence of the inhibitor, as described in Materials and Methods.

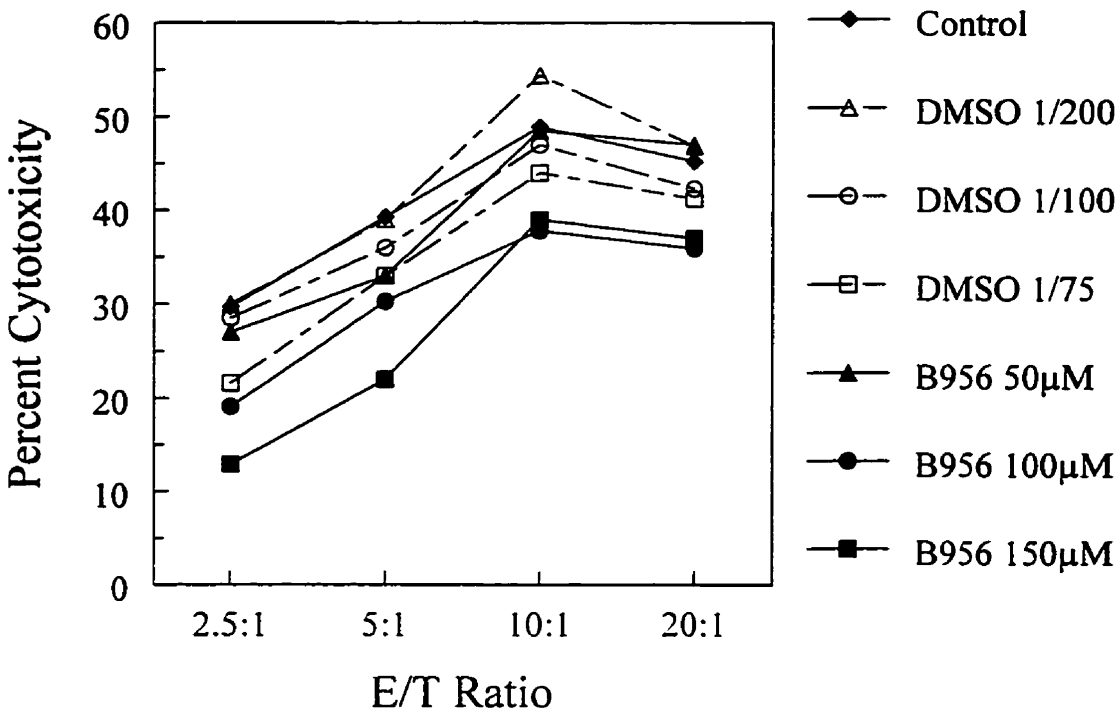

60
50
40
30
20
10
0
Percent Cytotoxicity
2.5:1
5:1
10:1
20:1
E/T Ratio
Control
DMSO 1/200
DMSO 1/100
DMSO 1/75
B956 50μM
B956 100μM
B956 150μM

Figure 4. Inhibition of NK3.3 cytotoxicity by the farnesyl transferase inhibitor B956 (lytic units). NK3.3 cells were treated with B956 at the concentrations indicated or DMSO as control, for 48h prior to assessment of cytotoxicity in a ^{51}Cr release assay in the presence of the inhibitor, as described in Materials and Methods. The effect of B956 on NK3.3 cytotoxic function expressed in lytic units (20%)/10^6cells +/- S.D. B956 was significantly different from DMSO control at concentrations of 100 μM and 150 μM ($P<0.02$).

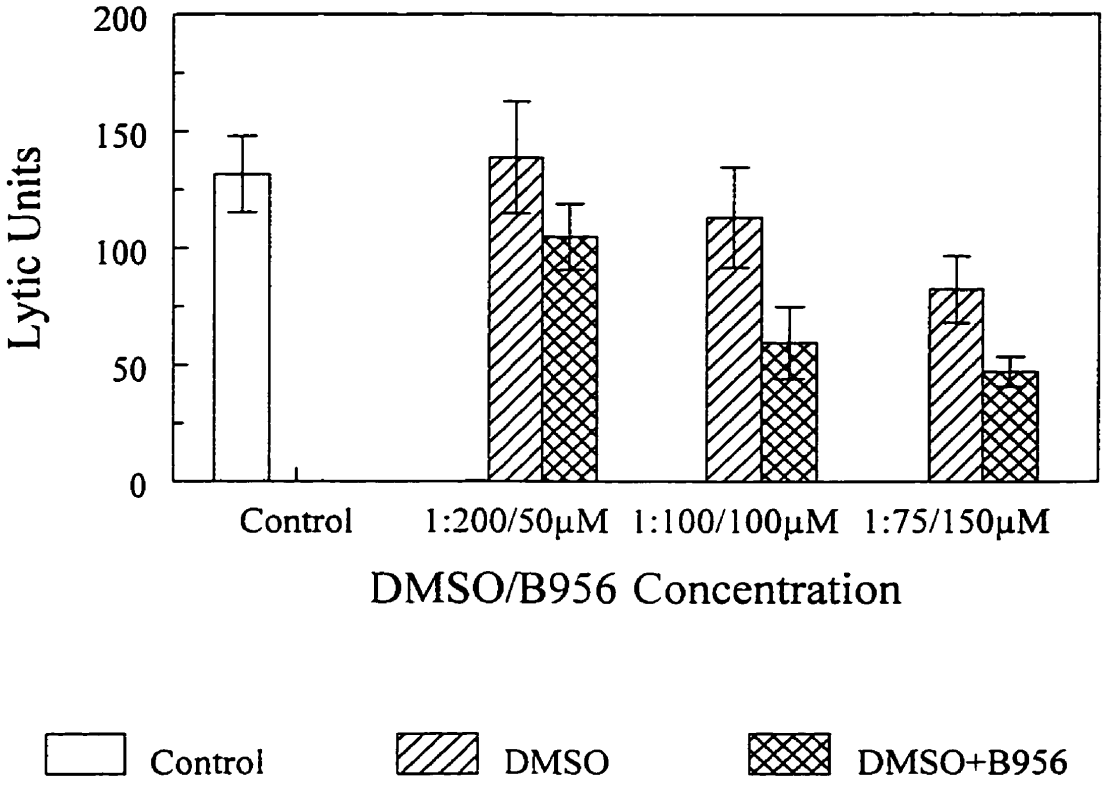

200
150
100
50
0
Lytic Units
Control
1:200/50μM
1:100/100μM
1:75/150μM
DMSO/B956 Concentration
Control
DMSO
DMSO+B956

Figure 5 shows the effect of B956 on NK3.3 cytotoxicity as a percent of DMSO control. The data were pooled from three independent experiments expressed as a percent of DMSO control to correct for experimental variation in cytotoxicity at different time points. It can be seen that treatment of NK3.3 with B956 at a concentration of 100 μM or150 μM resulted approximately in 50% inhibition of the cell mediated cytotoxicity as compared to the DMSO treated controls. Figures 6 and 7 describe the effect of B956 on PBL-NK cytotoxicity, expressed as percent cytotoxicity (Fig. 6) and as lytic units (Fig. 7). It can be seen in these figures that DMSO itself inhibited NK-mediated cytotoxicity to a great extent, making it impossible to observe an effect of B956.

In summary, the data presented in Figures 3-7 show that treatment of NK3.3 with the farnesyl inhibitor B956 resulted in significant inhibition of cell mediated cytotoxicity as compared to the DMSO treated controls, whereas any effect on PBL was masked by the action of the DMSO diluent. Further experiments were not possible because the company which supplied B956 as a gift could no longer do so.

Effect of the MMTV Promoter Inducer Dexamethasone on NK3.3 Cytotoxicity

As discussed previously, the inhibition of farnesyl transferase by B956 is not Ras specific and therefore it was desired to inhibit only the function of this protein. The H-Ras^{N-17} dominant negative mutant has been widely used for this purpose (Cai *et al.*, 1990). The available expression vector containing the gene coding for this mutant contains the MMTV promoter. This promoter is induced in the presence of dexamethasone. Figure 8 shows the effect of dexamethasone on NK3.3 cytotoxicity at concentrations and incubation times reflecting those necessary for induction of the MMTV promoter.

Figure 5. Percent inhibition of NK3.3 cytotoxicity by the farnesyl inhibitor B956 *versus* the DMSO control. NK3.3 cells were treated with B956 at the concentrations indicated or DMSO as control, for 48h prior to assessment of cytotoxicity in a ^{51}Cr release assay against K562 in the presence of the inhibitor, as described in Materials and Methods. The results are reported as mean percent cytotoxicity *vs* DMSO calculated as (Cytotoxicity B956)/ (Cytotoxicity DMSO) x 100.

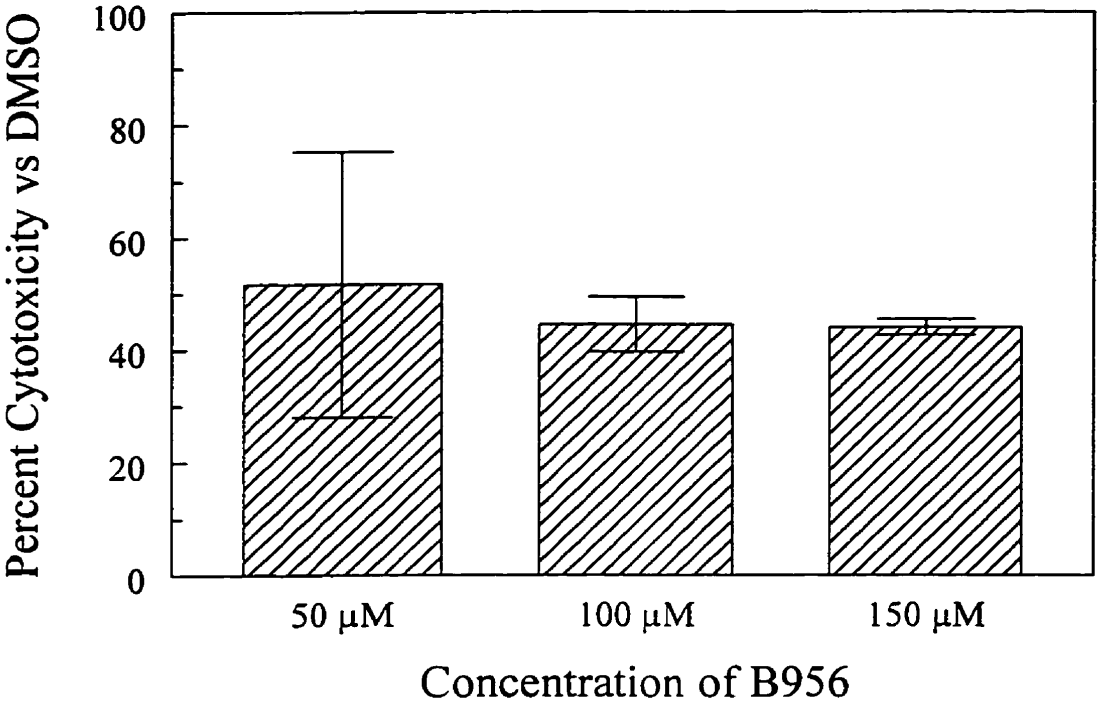

Percent Cytotoxicity vs DMSO
100
80
60
40
20
0
50 μM
100 μM
150 μM
Concentration of B956

Figure 6. Effect of the farnesyl inhibitor B956 on PBL-NK cytotoxicity (Chromium release). Peripheral blood was separated over Ficoll-Isopaque. Mononuclear cells were treated with B956 at the concentrations indicated or DMSO as control, for 48h prior to assessment of cytotoxicity in a ^{51}Cr release assay against K562, in the presence of the inhibitor, as described in Materials and Methods. Cytotoxicity is expressed as percent ^{51}Cr release.

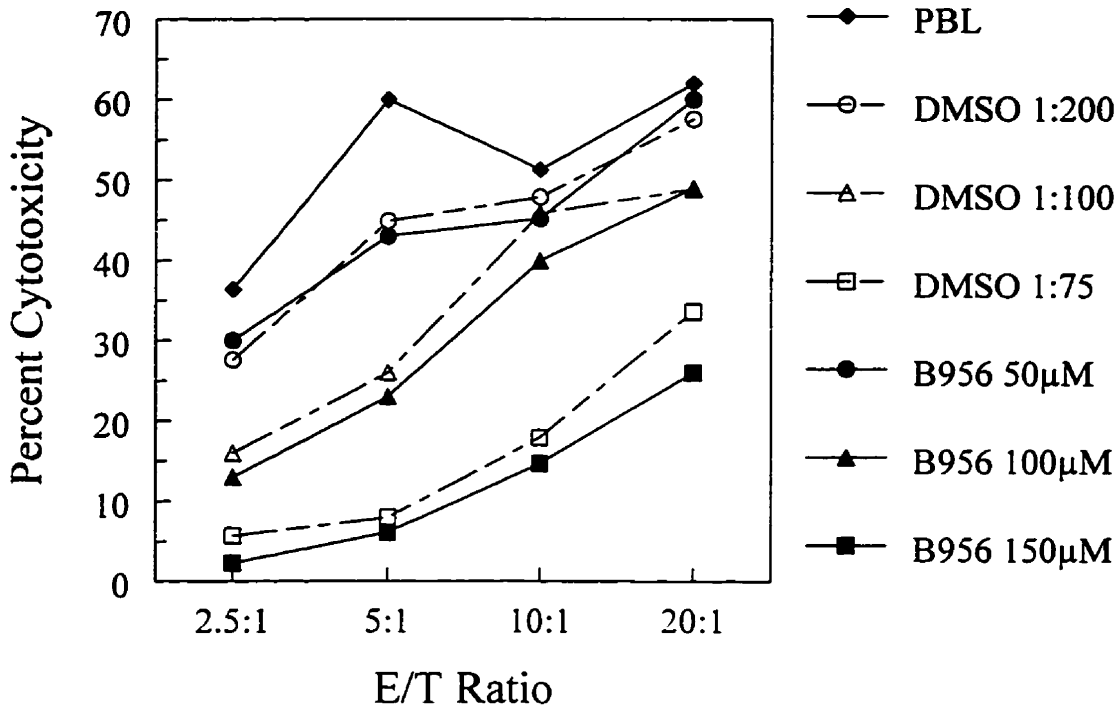

70
60
50
40
30
20
10
0
Percent Cytotoxicity
2.5:1
5:1
10:1
20:1
E/T Ratio
PBL
DMSO 1:200
DMSO 1:100
DMSO 1:75
B956 50µM
B956 100µM
B956 150µM

Figure 7. Effect of B956 on PBL-mediated cytolysis (lytic units). Peripheral blood was separated over Ficoll-Isopaque. Mononuclear cells were treated with B956 at the concentrations indicated or DMSO as control, for 48h prior to assessment of cytotoxicity in a ^{51}Cr release assay against K562, in the presence of the inhibitor, as described in Materials and Methods. Cytotoxicity is expressed in lytic units (20%)/10^6 cells +/- S.D. Both B956 and the DMSO control values were significantly lower than the untreated control ($p<0.05$), but not from each other.

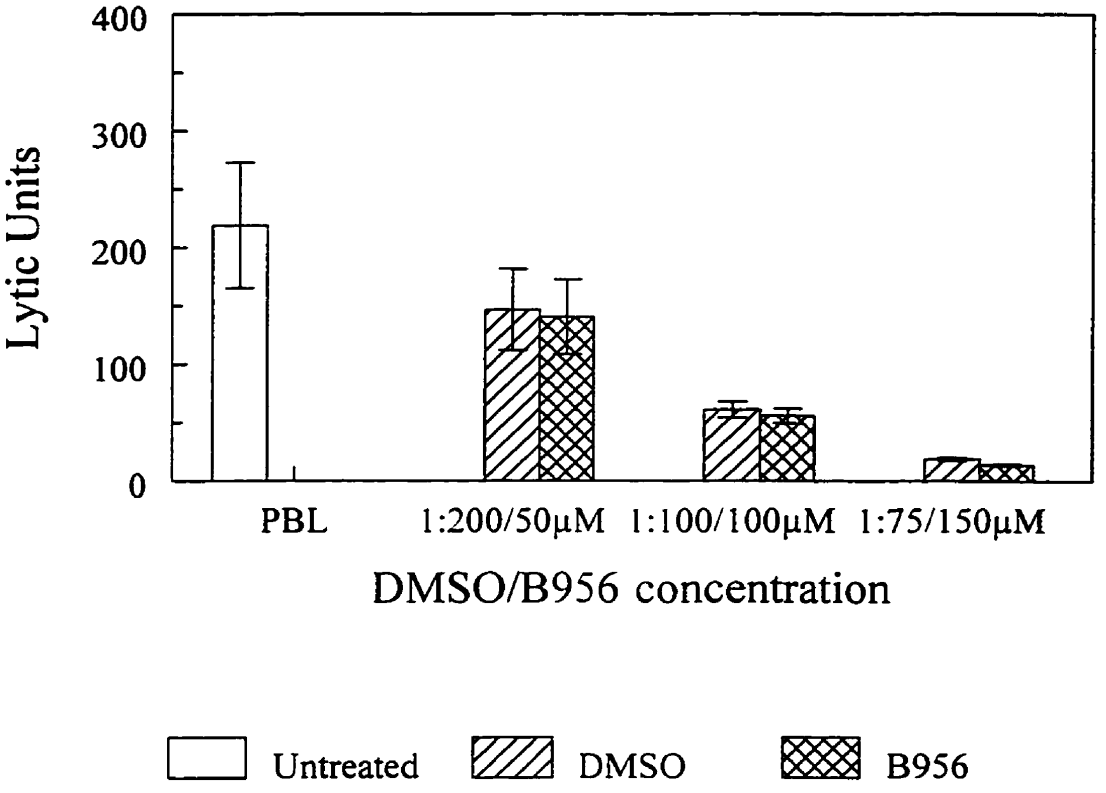
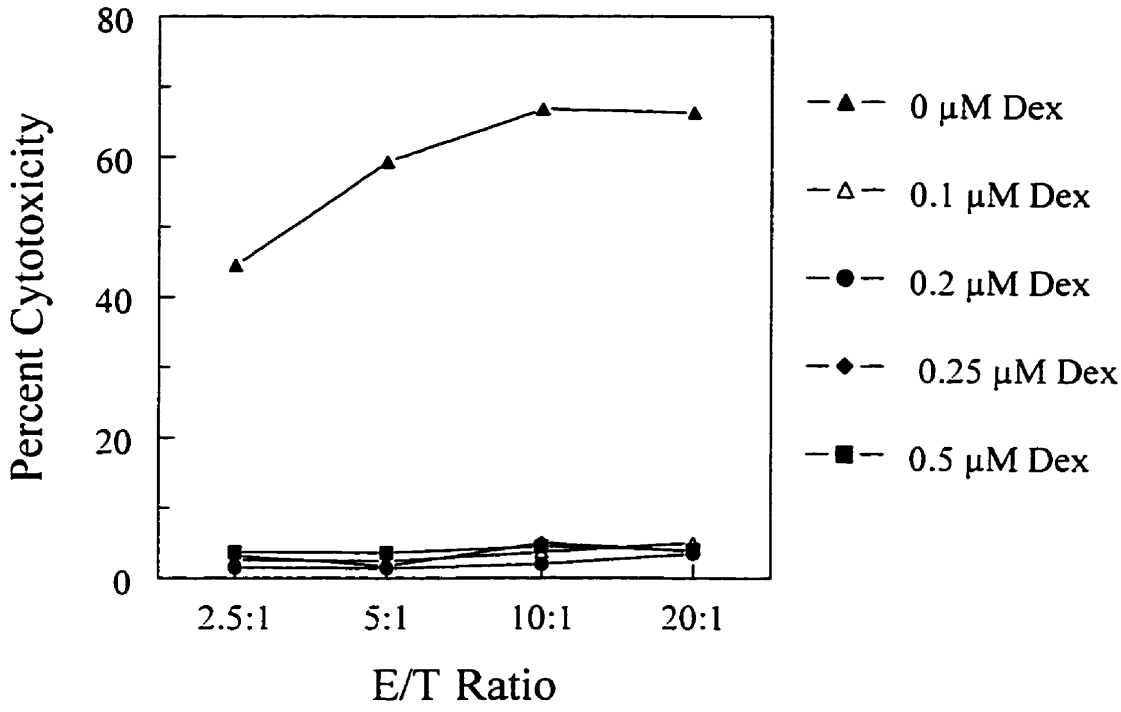

400
300
200
100
0
Lytic Units
PBL
1:200/50μM
1:100/100μM
1:75/150μM
DMSO/B956 concentration
Untreated
DMSO
B956

Figure 8. The effect of dexamethasone on NK3.3 cytotoxicity against K562. NK3.3 cells were treated with the indicated concentrations of dexamethasone for 72h prior to the assessment of the cytolytic function in an 18h ^{51}Cr release assay. Cytotoxicity is expressed as percent ^{51}Cr release. All concentrations were inhibitory ($p < 0.001$).

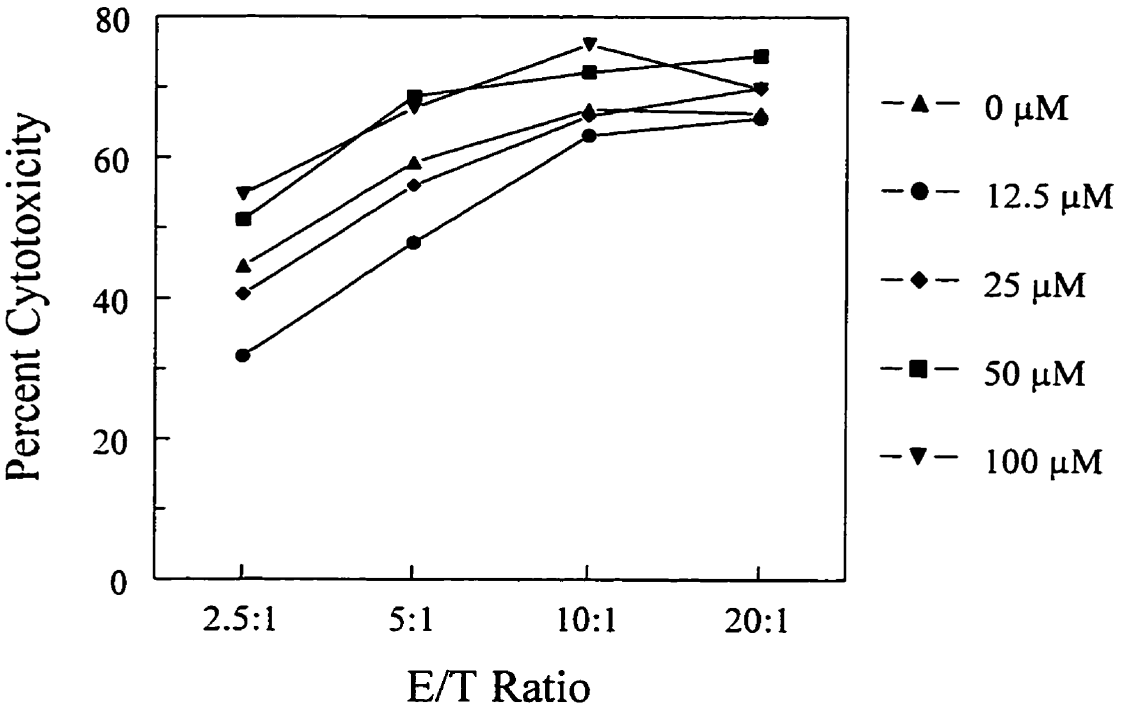

80
60
40
20
0
Percent Cytotoxicity
2.5:1
5:1
10:1
20:1
E/T Ratio
0 μM Dex
0.1 μM Dex
0.2 μM Dex
0.25 μM Dex
0.5 μM Dex

Treatment of the cells with dexamethasone at concentrations as low as 0.1 μM for 24h caused a complete inhibition of NK3.3 cytotoxicity. This result confirms the published results of the effect of dexamethasone on PBL-NK and T cell cytotoxic function (Migliorati *et al.*, 1994). Because of this inhibitory effect it was felt that the MMTV promoter could not be applied to the study of the effect of $p21^{Ras}$ inhibition on NK cell function.

Creation of a Vector Containing the Dominant Negative Mutant of Ras, Ras^{N-17}, under the Control of the Zn^{++} Inducible Promoter Rat MT-I

In order to avoid the use of dexamethasone, construction of a vector containing the N-17 mutant under the control of the Zn^{++} rat metallothionein-I (MT-I) promoter was attempted. Figure 9 shows the results of treatment of NK3.3 cells with Zn^{++} at the concentrations which would be used for the induction of the promoter. Concentrations of 12.5 and 25 μM were slightly inhibitory, while 50 and 100 μM were not significantly different than the controls.The Ras^{N-17} insert was released from the MMTV-N^{17} vector, which was obtained from Dr. Cooper (Dana Farber Institute, Boston) vector by digestion with the restriction enzyme *BamHI*, and isolated from an agarose gel as described in Materials and Methods. The recipient vector, (MT-I)-c-H-Ras, obtained from Dr. J. Filmus (University of Toronto), was digested with *BamHI* in order to release the c-H-Ras containing fragment. A fragment of 3.2 kb corresponding to the pBluescript vector containing the MT-I promoter was isolated from the gel and ligated to the 700 bp fragment containing the Ras^{N-17}. The ligation mixture was electroporated into electro-competent bacteria as described under the above. The orientation of the Ras^{N-17}

Figure 9. Effect of Zn^{++} on NK3.3 cytotoxicity against K562. NK3.3 cells were treated with the indicated concentrations of $ZnSO_4$ for 72h prior to the assessment of the cytolytic function in an 18h ^{51}Cr release assay. Cytotoxicity is expressed as percent ^{51}Cr release. None of the concentrations of $ZnSO_4$ were significantly inhibitory or stimulatory of the no $ZnSO_4$ control.


80
60
40
20
0
Percent Cytotoxicity
2.5:1
5:1
10:1
20:1
E/T Ratio
0 μM
12.5 μM
25 μM
50 μM
100 μM

containing fragment in the relationship to the MT-I promoter was checked by digestion with the restriction enzymes *Hind* III which cuts 160 bp from the start of the N-17 insert and *Xho* I which cuts in the vector's multiple cloning site just upstream of the MT-I promoter. If the correct orientation was achieved a fragment of 360 bp and a fragment of 3.6 kb would have been seen on a 1% agarose gel. On the other hand, if the wrong orientation was achieved a fragment of 560 bp and a fragment of 3.3 kb would be seen on the agarose gel. All clones obtained from several such ligations were in the wrong orientation, and the effort was abandoned.

Creation of NK Clones in which Ras has been Upregulated

The IL-2 receptor transmits its signals via several signal transduction pathways. One of them involves the STAT and the JAK proteins, and the other one involves the Ras signal transduction pathway. Crosslinking of CD16 activates a variety of signalling proteins such as PI-3 Kinase or PKC, and recently it has been reported that CD16 and IL-2 activate Ras in NK cells (Galandrini *et al.*, 1996). In order to single out the role of Ras in the cytolytic process, it was important to obtain NK cells expressing an activated mutant of Ras. Two different approaches were chosen in order to achieve this goal, one by transfection of NK3.3 cells with a vector containing an activated Ras mutant under the control of an inducible promoter, and second by expressing an activated Ras mutant constitutively.

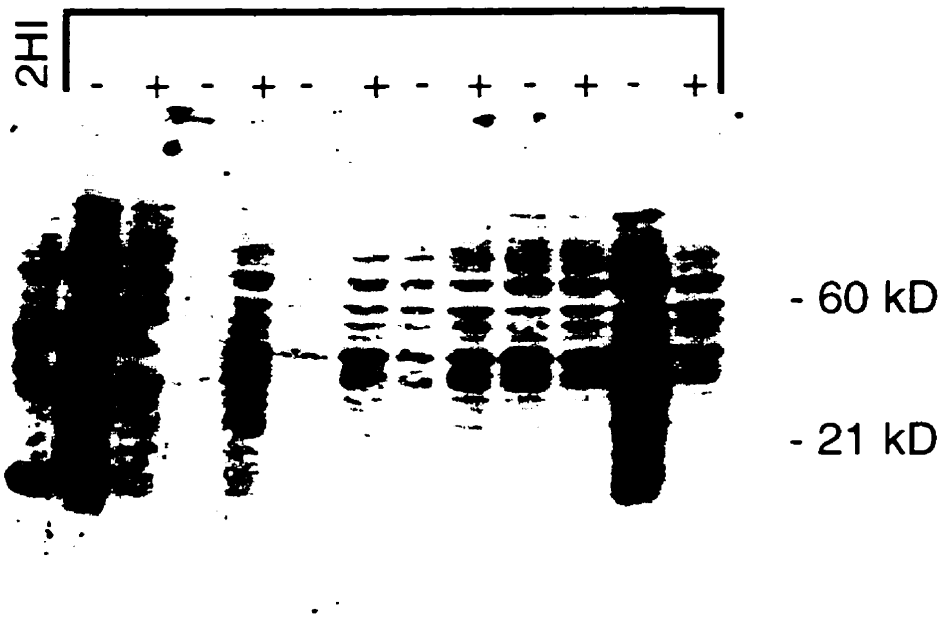
Creation of NK3.3 Clones Expressing Ras^{Leu-61} Under the Control of an Inducible Promoter

NK3.3 cells were transfected by electroporation using the Bio-Rad Gene Pulser, with a construct containing the active mutant H-Ras^{Leu-61} under the control of the rat metallothionein (MT-I) promoter, obtained from Dr. J. Filmus (University of Toronto), and cotransfected with DNA coding for G418 (geneticin) resistance, under the control of the phosphoglycerate kinase (PGK) promoter, obtained from Dr. J Rossant (University of Toronto). When transfected with constructs containing the MT-I promoter, regulation of Ras expression is achieved by the effect of positive induction by heavy metal inducers (Cd^{++} and/or Zn^{++}) (Rak *et al.*, 1995). Transfected cells were selected for G418 resistance and cloned by limiting dilution using irradiated Daudi cells as feeders (see Materials and Methods). A total of 424 G418 resistant clones derived from the NK3.3 parent clone were obtained. Some of these clones were tested for the MT-I promoter inducibility using various combinations of Zn^{++} and Cd^{++}. Even very small concentrations of Cd^{++} appeared to be toxic to the cells, and therefore Zn^{++} alone was used. The cells were induced with 100 μM-150 μM $ZnSO_4$ for 72h, the cells lysed, and the proteins subjected to SDS-PAGE electrophoresis, as described under Materials and Methods. The levels of $p21^{ras}$ were assessed by western blotting using the antibodies described below.

Assessment of $p21^{ras}$ by Western Blot using Pan-Ras Ab-1

The first antibody used was the antibody pan Ras Ab-1 (Oncogene Research products, Cambridge MA). Figure 10 shows a western blot of lysates from NK3.3 transfected with the construct described above using the pan-ras Ab-1 for the detection of

Figure 10. Western blotting analysis of Ras expression in NK3.3 clones transfected with the MT-Ras construct. MT-Ras-NK3.3 clones were treated with 100 μM Zn^{++} for 72h, then the cells were lysed as described in the Materials and Methods.10 mg protein were loaded in each lane on a 12% SDS-PAGE. Proteins were transferred to nitrocellulose and immunobloted with pan-ras Ab-1 to detect total Ras protein. Every two lanes represent a pair of untreated and Zn^{++} treated cells derived from one clone. (+) = + Zn^{++}, (-) = no inducer. 1 – 6 = MT-Ras-NK3.3 clone number (chosen arbitrarily). Lane 13 – 2H1.


2HI
- + - + - + - + - + - +
- 60 kD
- 21 kD

$p21^{ras}$. A strong band can be seen corresponding to a protein of molecular weight of 60 kD rather than 21 kD which is the molecular weight corresponding to Ras. The specificity, or lack of it, was later acknowledged by Oncogene Research Products.

Assessment of $p21^{ras}$ by Western Blot using Pan Ras Ab-3

A second antibody, pan Ras Ab-3, was recommended and sent by Oncogene Research Products, as being specific for $p21^{ras}$. G418 resistant NK3.3 clones which had been transfected with Ras^{Leu-61} were lysed and 15 µl from the 1 ml lysates were run on a SDS-PAGE gel. The proteins were then transferred to nitrocellulose and immunoblotted with pan Ras Ab-3. Blotting with this antibody resulted in a strong single band of 21 kD molecular weight (Fig. 11), which was strong in every cell line tested, including an insect derived cell line SF-9 (obtained from Dr. E. Carstens) used as a negative control (data not shown). $p21^{ras}$ is not abundant in the cell and this result led us to question the specificity of this antibody also. The antibody Y13-259 is a pan Ras antibody which recognises all Ras proteins i.e., K-Ras, H-Ras, N-Ras A and N-Ras B. This antibody is widely used for the immunoprecipitation of $p21^{ras}$ and its specificity was not questionable. Therefore, this antibody was used for testing the specificity of the antibody pan Ras Ab-3. For this purpose, the same cell lysates previously used for western blots with pan Ras Ab-3 were split into two separate microcentrifuge tubes, and $p21^{ras}$ was immunoprecipitated using the antibody Y13-259. The immunoprecipitates were then subjected to electrophoresis on SDS page, transferred to nitrocellulose and blotted with the same antibody (Y13-259) or with pan Ras Ab-3. Figure 12 shows a western blot using the antibody Y13-259 for immunoprecipitation and immunoblotting. A band

Figure 11. Western blotting analysis of Ras expression using pan-ras Ab-3, in NK3.3 clones transfected with the MT-Ras construct. MT-Ras-NK3.3 clones were treated with 100 μM Zn^{++} for 72h, then the cells were lysed as described in the Materials and Methods.10 μg protein were loaded in each lane on a 12% SDS-PAGE. Proteins were transferred to nitrocellulose and immunobloted with pan-ras Ab-3 to detect total Ras protein. Every two lanes represent a pair of untreated and Zn^{++} treated cells derived from one clone. (+) = + Zn^{++}, (-) = no inducer. 1 – 6 = MT-Ras-NK3.3 clone number (chosen arbitrarily). Lane 13 – 2H1.

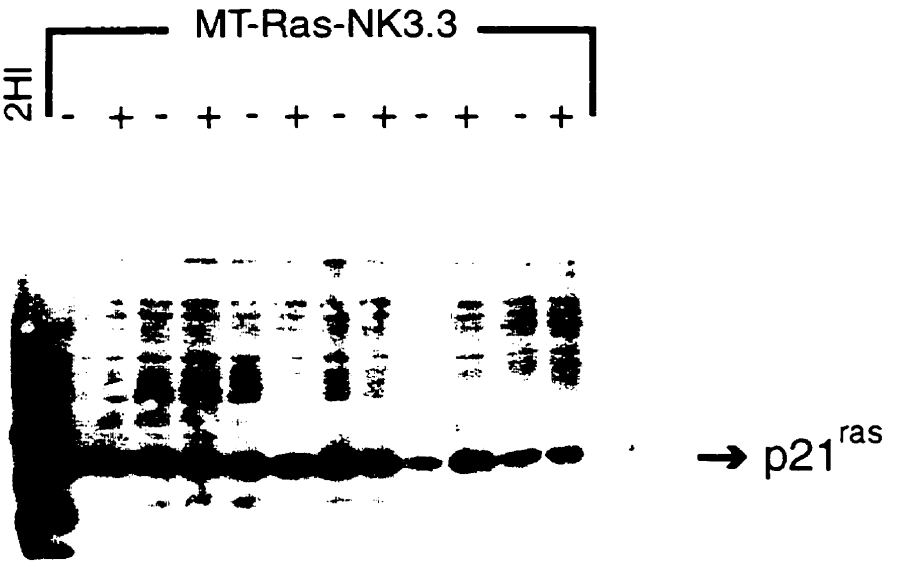
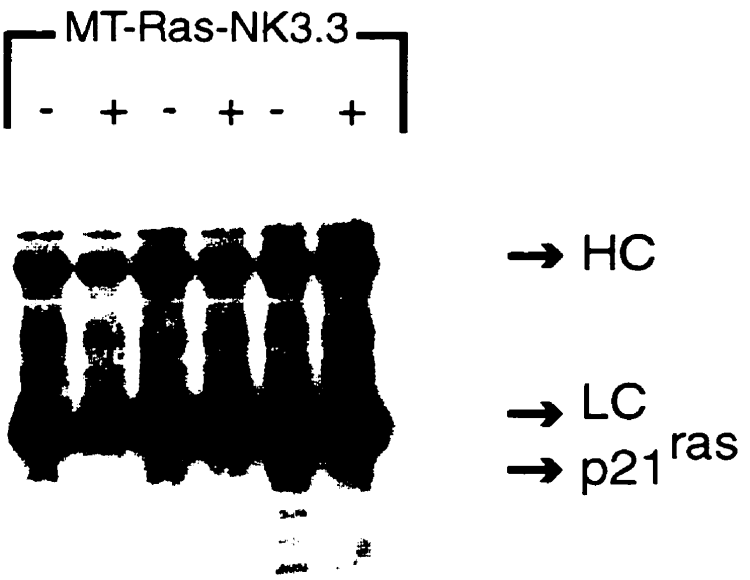

MT-Ras-NK3.3
2HI
- + - + - + - + - + - +
→ p21ras

Figure 12. Expression of $p21^{ras}$ in MT-Ras-NK3.3 clones, using immunoprecipitation and immunoblotting with pan-ras Ab3. NK3.3 cells previously transfected with the MT-H-Ras construct, were induced for 72 h with 100 μM Zn^{++}, and lysed as described in Materials and Methods. Ras proteins were immunoprecipitated with the broadly rective antibody Y13-259. The immunoprecipitates were then immunoblotted with pan-ras Ab-3. 1 – 3 = MT-Ras-NK3.3 clone number. (+) = Zn^{++} induced. (-) = NK3.3 clones cultured in the absence of the inducer. LC, light chain of the immuprecipitating Y13-259 antibody (25 kD). HC, heavy chain of the immunoprecipitaing antibody (45 kD).


MT-Ras-NK3.3
- + - + - +
HC
LC
p21ras

corresponding to the light chain and another to the heavy chain of the antibody used for the immunoprecipitation can be visualised. In addition a band corresponding to $p21^{ras}$ can be seen. Figure 13 shows the results from a western blot using Ab-3 for the immunoblotting. Again, the heavy chain and the light chain are visible and a weak band of 21 kD corresponding to $p21^{ras}$ can be seen. The results in Figures 12 and 13 show that unless only a minuscule fraction of the $p21^{ras}$ present in the cell is immunoprecipitated, the band seen in Fig. 11 does not correspond entirely to $p21^{ras}$, but to another protein of the same molecular weight which is abundant in the cell.

Assessment of $p21^{ras}$ by Western Blot using Pan Ras Ab-2

Finally, a third antibody, pan Ras Ab-2 (Oncogene Research Products), was used. Figure 14 shows a western blot using this antibody. Binding of pan Ras Ab-2 to the protein resulted in a very faint band corresponding to a molecular weight of 21 kD in lysates of the NK3.3 derived clones. As a positive control the 2H1 cell line was used. 2H1 is a mouse fibroblast cell line transfected with Ras $^{Arg\text{-}12}$ under the control of the mouse metallothionein promoter. An increase in Ras expression could be visualised when 2H1 cells were treated with Zn^{++} for the induction of the mouse metallothionein promoter. These data indicated that pan Ras Ab-2 could be used for the visualisation of mutant Ras in the transfected clones. Fifty of the 424 G418-resistant NK3.3 clones co-transfected with H-$Ras^{Leu\text{-}61}$, described above, were assessed for mutant $p21^{ras}$ expression using the antibody pan ras Ab-2. None of these clones was found to express the mutant. It is clear that by co-transfecting with two separate vectors, we obtained a large proportion of NK3.3 clones which expressed the G418 resistance gene, but did not express the gene

Figure 13. Expression of $p21^{ras}$ in MT-Ras-NK3.3 clones, using immunoprecipitation and immunoblotting with Y13-259. NK3.3 cells previously transfected with the MT-H-Ras construct, were induced for 72 h with 100 μM Zn^{++}, and lysed as described in Materials and Methods. Ras proteins were immunoprecipitated with the broadly reactive antibody Y13-259. The immunoprecipitates were then immunoblotted with the same antibody. 1 – 3 = MT-Ras-NK3.3 clone mumber. (+) = Zn^{++} induced. (-) = NK3.3 clones cultured in the absence of the inducer. LC, light chain of the immuprecipitating Y13-259 antibody (25 kD). HC, heavy chain of the immunoprecipitaing antibody (45 kD).

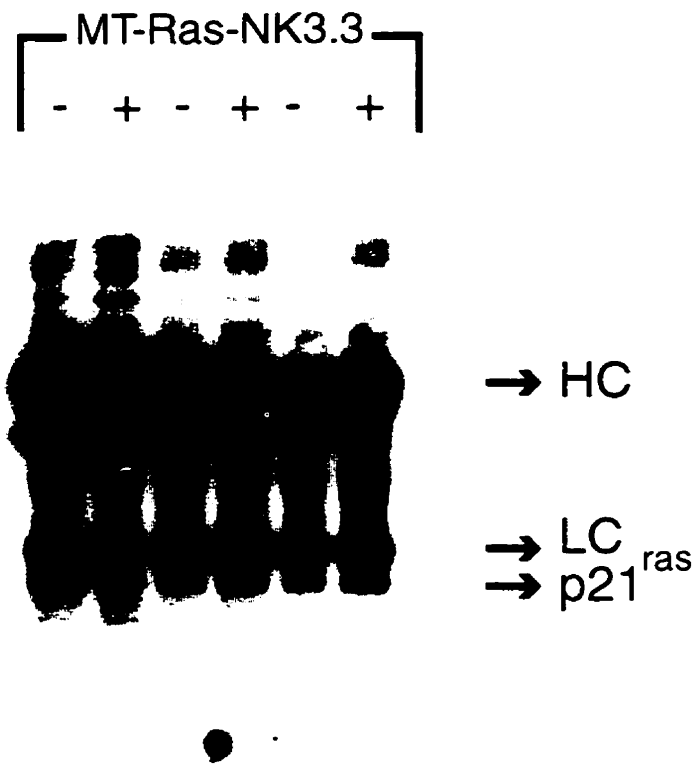
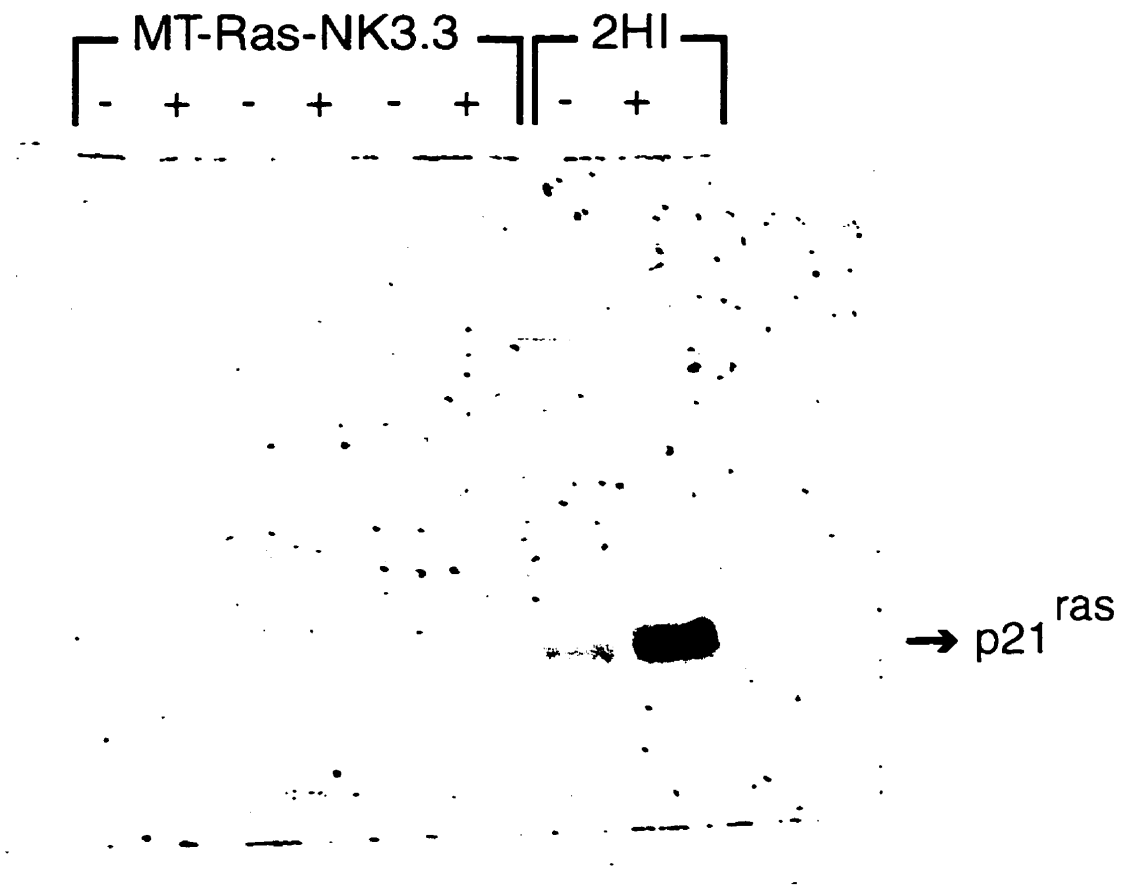

MT-Ras-NK3.3
- + - + - +
HC
LC
p21ras

Figure 14. Western blotting analysis of Ras expression using pan-ras Ab-2, in NK3.3 clones transfected with the MT-Ras construct. MT-Ras-NK3.3 clones or 2H1 cells were treated with 100 μM Zn^{++} for 72h, then the cells were lysed as described in the Materials and Methods.10 μg protein were loaded in each lane on a 12% SDS-PAGE. Proteins were transferred to nitrocellulose and immunobloted with pan-ras Ab-2 to detect total Ras protein. Every two lanes represent a pair of untreated and Zn^{++} treated cells derived from one clone. (+) = + Zn^{++}, (-) = no inducer. 1 – 5 = MT-Ras-NK3.3 clone number (chosen arbitrarily). Lane 13 – 2H1 cultured in the absence of inducer. Lane 14 – 2H1 cells induced with Zn^{++}.


MT-Ras-NK3.3
2HI
- + - + - + - +
p21 ras

of interest, H-Ras^{Leu-61}. Due to time and cost constraints we decided to freeze the unscreened clones and attempted a second approach for the stable expression of the gene in NK3.3 cells.

Creation of a Vector Containing the H-Ras^{Leu-61} and G418 Resistance

It is clear that by transfection with two separate vectors we obtained a large proportion of clones that expressed the geneticin resistance gene but did not express the gene of interest, in this case H-Ras^{Leu-61}. In view of the results using co-transfection of G418 and H-Ras^{Leu-61}, we decided to create a vector that contains both the gene encoding for resistance to G418 and H-Ras^{Leu-61} on the same vector. The expression of the G418 resistance would be driven by the powerful phosphoglycerate kinase (PGK) promoter, and H-Ras^{Leu-61} expression would be controlled by the metallothionein promoter. These cells would be G418 resistant, but the expression of H-Ras^{Leu-61} would be inducible by the presence of Zn^{++}. The (MT-I)-c-H-Ras vector was digested with *Ssp* I in order to create compatible sites for the neo cassette which was released from the PGK-neo vector by digestion with the restriction enzymes *Ssp* I and *Sma* I. Lack of adequate information from the laboratory which created the vector, made the digestion of the (MT-I) with enzymes that would create compatible sites for the neo cassette an impossible task, since additional sites for the *Ssp* I enzyme were discovered upon digestion.

Creation of Clones Expressing the Constitutively Active Ras Mutant H-Ras^{Arg-12}

Due to the difficulties encountered with the transfection and screening of the clones expressing the H-Ras^{Leu-61} under the control of the inducible rat MT-I promoter we

decided to use a different method for delivering the gene of interest into NK3.3 cells. The method of choice was the use of retroviral transduction. For this purpose, we used a packaging line (PG13), which produces a high titer of a retrovirus derived from the gibbon ape leukemia virus, capable of infecting human cells. This vector contains the H-v-Ras$^{Arg\text{-}12}$ under the control of the murine stem cell virus promoter (MSCV) long terminal repeat (LTR) and the neo resistance gene driven by the phosphoglycerate kinase (PGK) promoter (obtained from Dr. J. Dick, Dept. Of Human Genetics, University of Toronto). The vector has been previously described (Hawley *et al.*, 1995). To generate neo controls, the cells were cocultured with the same packaging line producing a retrovirus which contains only the neomycin resistance but not the activated Ras mutant. The infection was performed by coculturing the NK3.3 clones with the retrovirus producing cell line for 72h (in Dr. J. Dick's Level 3 laboratory). Three such infections were performed, two of them in the presence of IL-2 and one of them in the absence of the cytokine. The cells were screened for the presence of helper virus using HeLa cells as recipients, as described in Materials and Methods. Briefly, supernatants from NK3.3 cells previously infected with the retrovirus were collected and applied onto HeLa cells for 48h. Then the HeLa cells were screened for G418 resistant colonies over a period of two weeks. No colonies were obtained, indicating that it was safe to culture the NK3.3 cells in a Level 2 facility. Infected NK3.3 cells were selected for G418 resistance for two weeks, resulting in several G418 resistant cell lines obtained from independent infections. We refer to these cell lines as NK3.3R1, R2 and R3. NK3.3R3 was the cell line infected with the retrovirus in the absence of IL-2 and cultured in the absence of the cytokine for another week. By that point it was observed that NK3.3R3 was not proliferating well in

the absence of IL-2, and thereafter the cytokine was added to the medium. All three sublines were maintained in G418 in order to avoid the loss of the gene as a result of removing the selective agent. The NK3.3R3 cell line was subcloned further for reasons described below. We obtained 25 such subclones. Since different levels of expression could be obtained depending on the number of copies integrated in the genome, the subclones were retained for further characterization, if necessary. This thesis focuses on the three cell lines originally obtained.

Expression of v-H-Ras^{Arg-12} in the G418 Resistant Cell Lines NK3.3R1, R2 and R3

The expression of H-Ras^{Arg-12} and the expression of the G418 resistance are controlled by two different promoters. The G418 resistance is controlled by the PGK promoter, while the Ras gene is controlled by the MMSV promoter. It is not inconceivable that the G418 resistance would be expressed and H-Ras^{Arg-12} would be lost or not expressed, if it did not confer the cells with any selective benefit. Furthermore it has been reported that IL-2 and CD16 costimulation causes NK cells to undergo apoptosis (Ortaldo *et al.,* 1995). Since CD16 crosslinking activates Ras in NK cells and the cells are cultured in IL-2, the combination of IL-2 and expression of activated Ras may have had the same effect, leading to the loss of the cells that express the activated form of Ras. In order to establish v-H-Ras^{Arg-12} expression, v-H-Ras mRNA levels, as well as the protein levels of the Arg-12 mutant were studied in each cell line.

As described in Materials and Methods, total RNA was extracted, from NK3.3, NK3.3neo, NK3.3R1, NK3.3R2 and NK3.3R3 using TRizol. The RNA was run on an agarose formaldehyde gel, transferred to a nylon membrane and probed with a ^{32}P-

labelled v-H-Ras specific probe, obtained by PCR from the vector used for the infection (Hawley *et al.*, 1995). Figure 15 shows a Northern Blot of the total RNA from the NK3.3 cell lines probed with a probe specific for v-H-Ras and a probe specific for the house keeping gene GAPDH to correct for differences in the amounts of RNA loaded. A band corresponding to RNA of a size of 3.6 kb was found in NK3.3R1 and R2. Interestingly, an additional band of 2.9kb could be seen only in the NK3.3R3 cell line. The level of expression was comparable between NK3.3R1 and R2 but was lower in NK3.3R3 despite equal amounts of RNA loaded. v-H-Ras$^{Arg\text{-}12}$ expression was assessed at the protein level as well, in order to establish that proper translation from mRNA to protein had occurred. For this purpose the cells were lysed in lysis buffer and p21^{ras} was immunoprecipitated with the antibody Y13-259 as described in Materials and Methods. The immunoprecipitate was run on a 12% SDS-PAGE gel and blotted with an antibody specific for the Arg-12 mutant. All cell lines in which the activated form of ras was inserted expressed the mutated protein (Fig. 16).

Figure 15. Northern blot analysis of total RNA from MSCV-v-H-Ras^{Arg-12} infected NK3.3 cell lines. Total RNA was extracted from NK3.3 cell lines. 10 μg total RNA were loaded in each lane. The RNA was transferred to a nylon membrane and hybridized with probes for v-H-Ras and for glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The size of the transcripts corresponding to v-H-ras (3.6 kb and 2.9 kb) and to GAPDH (1.4 kb) are indicated in the Figure.

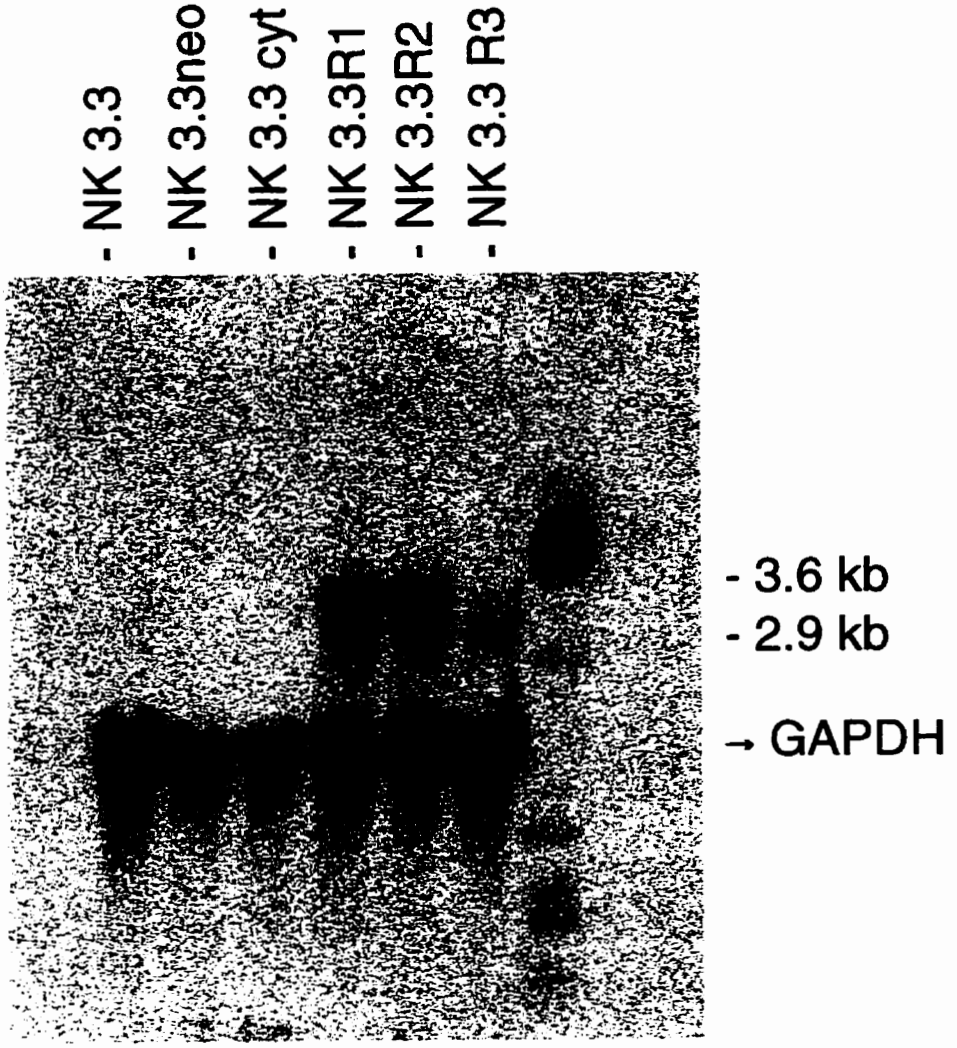

- NK 3.3
- NK 3.3neo
- NK 3.3 cyt
- NK 3.3R1
- NK 3.3R2
- NK 3.3 R3
- 3.6 kb
- 2.9 kb
→ GAPDH

Figure 16. Expression of Ras^{Arg-12} in the NK3.3 derived cell lines. NK3.3 cells previously infected with the MSCV-v-H-Ras vector, lysed as described in Materials and Methods. Ras proteins were immunoprecipitated with the broadly rective antibody Y13-259. The immunoprecipitates were then immunoblotted with the Arg 12 specific antibody pan-Ras^{Arg-12}. LC, light chain of the immuprecipitating Y13-259 antibody (25 kD). HC, heavy chain of the immunoprecipitaing antibody (45 kD).

NK3.3
NK3.3neo
NK3.3R1
NK3.3R2
NK3.3R3
HC
LC
Ras
Arg-12

II. Characterisation of NK3.3 Clones Expressing the Ras Oncogene

Cytotoxic activity of the NK3.3R1, R2, R3 *versus* NK3.3 controls

Ras is activated by a variety of extracellular stimuli such as binding of IL-2 to its receptor and CD16 crosslinking. Natural killer cells are activated by the binding of the Fc part of IgG antibodies to the FcRIII (CD16) and by IL-2 (Trinchieri, 1989). The IL-2 activation results in a variety of intracellular and extracellular changes leading to an increase in the cytolytic function and in the range of target cells killed by these lymphokine activated killer cells (LAK). Therefore, the effect of the constitutive activation of Ras on the cytolytic function of NK3.3 was investigated. For this purpose NK3.3, the original clone, NK3.3 neo, the cells infected with the vector containing only the G418 resistance, and NK3.3R1, R2, and R3 were tested in an 18h ^{51}Cr release assay against the NK-sensitive K562 cell line and the NK-resistant Raji cell line.

Figures 17 and 18 show the results of cytotoxicity assays performed shortly after (2.5 months) the v-H-Ras^{Arg-12} expressing NK3.3 cell lines were obtained. As illustrated previously (Fig. 1), the cytolytic function of NK3.3 decreased with time in culture, until, after a period of several months, although viable and proliferating vigorously, the cells lost almost all capability of killing the NK-sensitive cell line K562. The results in Figure 17 (expressed as percent chromium release), show that the NK3.3R1, R2 and R3 sublines are more cytotoxic than the NK3.3 parental line. Figure 18 shows the same data presented as lytic units for curve comparison. Interestingly, the subline NK3.3R3 exhibits the highest cytotoxicity, as well as the lower v-H-Ras^{Arg-12} expression (see Fig.15). Figures 19 and 20 show the cytotoxicity of the NK3.3 cell lines after long term culture (8 months), presented as percent cytotoxicity (Fig. 19) and as lytic units (Fig. 20). It can be seen from

Figure 17. Cytotoxicity of NK3.3R1 NK3.3R2 and NK3.3R3 *versus* NK3.3 control (Chromium release). NK3.3 cell lines were maintained in culture for 2.5 months, prior to the assessement of the cytotoxic function. The cells were stimulated with 100 U/ml IL-2 prior to analysis for cytolytic activity in an 18h ^{51}Cr release assay against the NK-sensitive target K562, as described in Materials and Methods. Cytotoxicity is expressed as percent ^{51}Cr release.

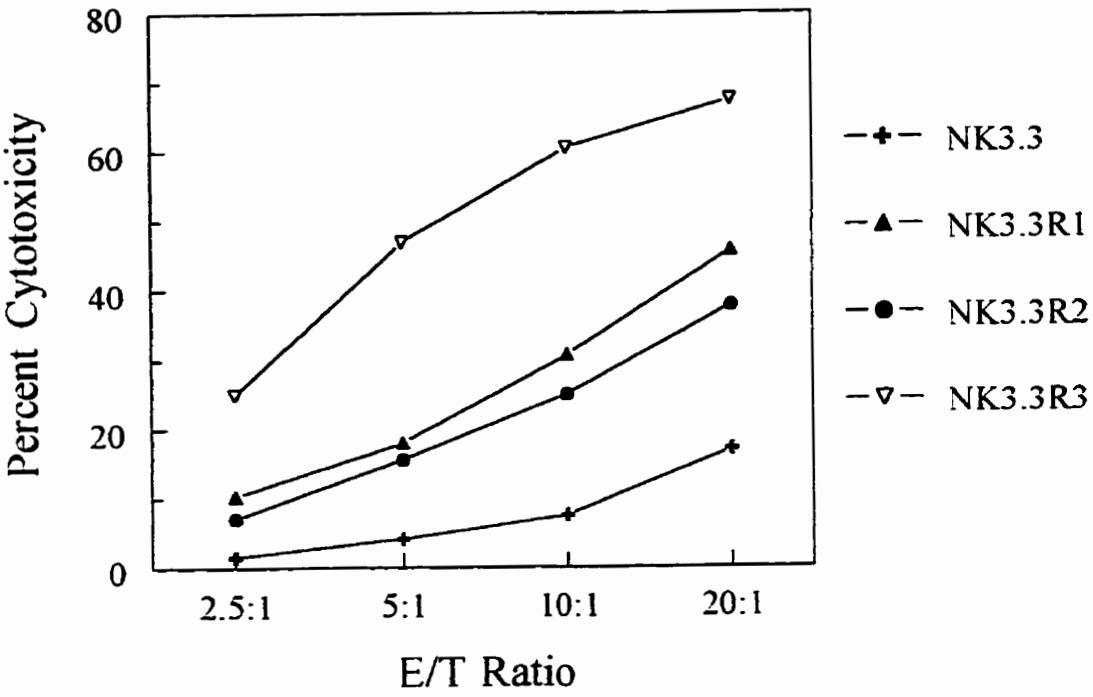

80
60
40
20
0
Percent Cytotoxicity
2.5:1
5:1
10:1
20:1
E/T Ratio
NK3.3
NK3.3R1
NK3.3R2
NK3.3R3

Figure 18. Cytotoxicity of NK3.3R1 NK3.3R2 and NK3.3R3 *versus* NK3.3 control. NK3.3 cell lines were maintained in culture for 2.5 months, prior to the assessement of the cytotoxic function. The cells were stimulated with 100 U/ml IL-2 prior to analysis for cytolytic activity in an 18h ^{51}Cr release assay against the NK-sensitive target K562, as described in Materials and Methods. Cytotoxicity is expressed in lytic units. All of the Ras lines are significantly more cytotoxic than the NK3.3 control ($p<0.025$). NK3.3 Ras3 is significantly more cytotoxic than the other two NK3.3 Ras sublines ($p<0.01$).

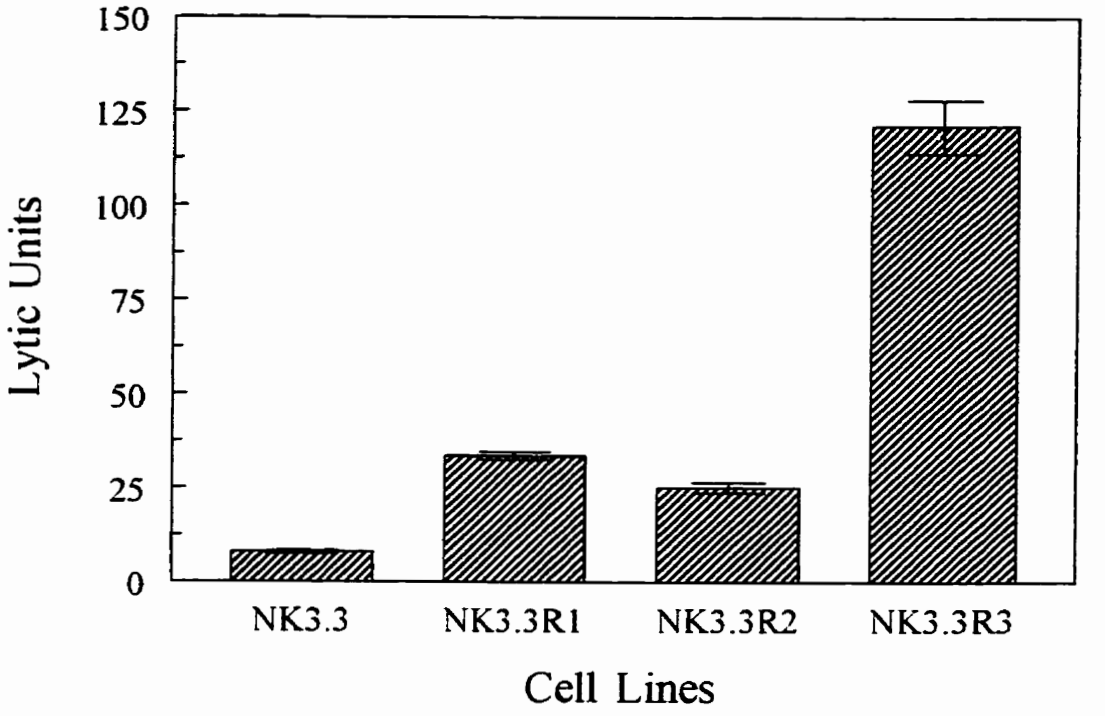

150
125
100
75
50
25
0
Lytic Units
NK3.3
NK3.3R1
NK3.3R2
NK3.3R3
Cell Lines

Figure 19. Cytotoxicity of NK3.3R1, NK3.3R2, and NK3.3R3 *versus* NK3.3 controls after long term in culture. NK3.3 cell lines were maintained in culture for 8 months, prior to the assessement of the cytotoxic function. The cells were stimulated with 100 U/ml IL-2 prior to analysis for cytolytic activity in an 18h ^{51}Cr release assay against the NK-sensitive target K562, as described in Materials and Methods. Cytotoxicity is expressed as percent ^{51}Cr release.

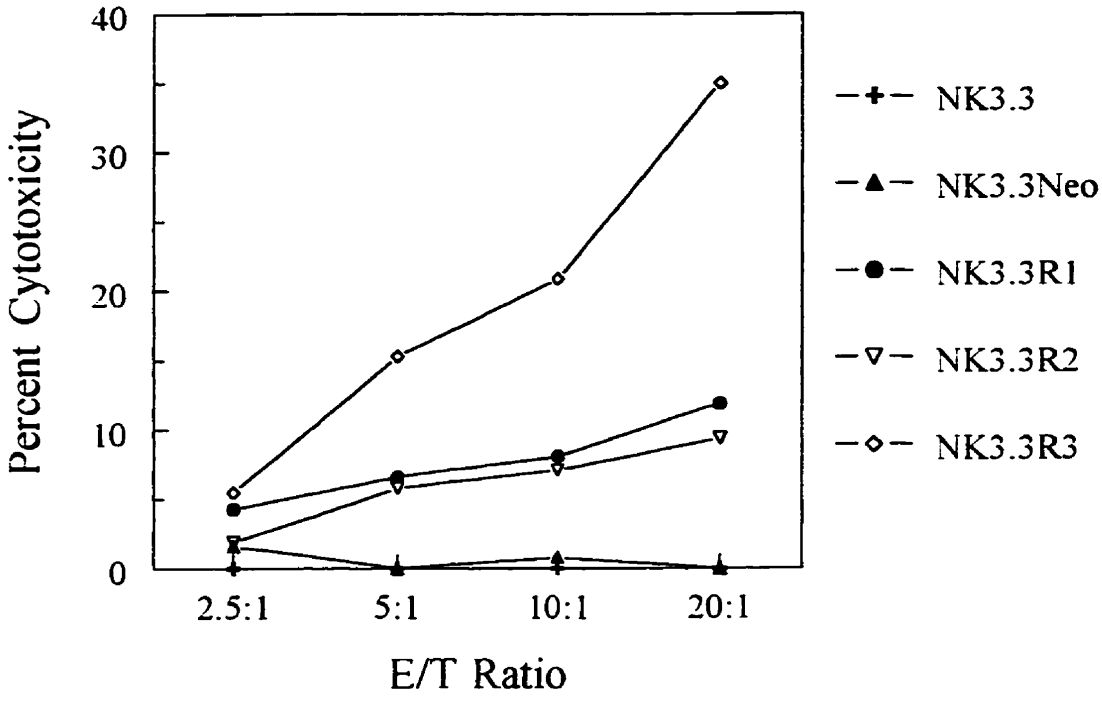
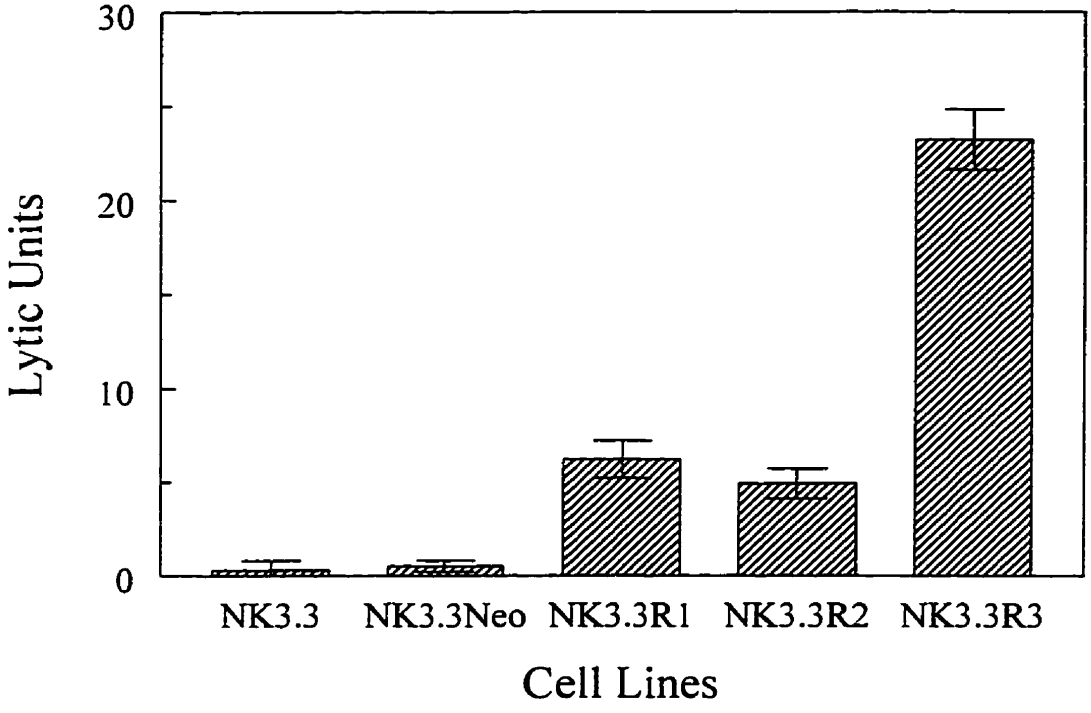

40
30
20
10
0
Percent Cytotoxicity
2.5:1
5:1
10:1
20:1
E/T Ratio
NK3.3
NK3.3Neo
NK3.3R1
NK3.3R2
NK3.3R3

Figure 20. Cytotoxicity of NK3.3R1, NK3.3R2, and NK3.3R3 *versus* NK3.3 controls after long term in culture. NK3.3 cell lines were maintained in culture for 8 months, prior to the assessement of the cytotoxic function. The cells were stimulated with 100 U/ml IL-2 prior to analysis for cytolytic activity in an 18h ^{51}Cr release assay against the NK-sensitive target K562, as described in Materials and Methods. Cytotoxicity is expressed lytic units. NK3.3 R1 and NK3.3R2 are significantly more cytotoxic than controls ($p<0.03$). NK3.3 R3 cytotoxicity was significantly greater than all other sublines ($p<0.05$ by non-overlapping 95% confidence intervals).


30
20
10
0
Lytic Units
NK3.3
NK3.3Neo
NK3.3R1
NK3.3R2
NK3.3R3
Cell Lines

these figures that the controls, NK3.3 and NK3.3neo, have lost their cytolytic function but the three Ras^{Arg-12} expressing cell lines maintain their cytotoxicity against the cell line K562. Once more, the same pattern can be seen, *i.e.*, that the NK3.3R3 cell line exhibits the highest cytotoxicity *versus* K562. These results indicate that constitutive activation of Ras increases the cytotoxic function of NK3.3 *versus* K562.

The next step was to investigate whether the v-H-Ras^{Arg-12} expressing cell lines exhibit a LAK-like phenotype. For this purpose the cytotoxic function of the cells *versus* the NK-resistant but LAK-sensitive cell line Raji was investigated. Figures 21 and 22 show that NK3.3R1 and NK3.3R3, but not NK3.3R2, have a higher cytotoxicity against the NK-resistant cell line Raji, than NK3.3 controls, although not as high as fully activated NK cells from PBL.

From the results described above, it was concluded that the expression of a constitutive form of Ras changes the phenotype of NK3.3 into a more cytotoxic cell line against K562 and Raji. Furthermore, as opposed to the NK3.3 cell line, these cell lines maintain their cytotoxic ability for a much longer period of time as compared to controls. It was decided to investigate how the phenotype of these cell lines changed, and what the role of Ras might be in the creation of this phenotype.

Proliferation of NK3.3R1, R2 and R3, in Response to Cytokines

One of the first things investigated was the response to cytokines of the cell lines expressing the activated form of Ras. Most cytokines transmit their signals via the activation of Ras (Ogata *et al.*, 1997; Knall *et al.*, 1996; Pazdrak *et al.*, 1995; Graves *et al.*, 1992). In fact, only a few cytokines, such as IL-4, do not activate Ras when bound to

Figure 21. LAK activity of NK3.3R1, NK3.3R2 and NK3.3R3 *versus* NK3.3 controls (Chromium release). NK3.3 derived cell lines were stimulated with 100 U/ml IL-2 prior to analysis for cytolytic activity in an 18h ^{51}Cr release assay against the NK-resistant and LAK-sensitive target Raji, as described in Materials and Methods. Cytotoxicity is expressed as percent ^{51}Cr release.

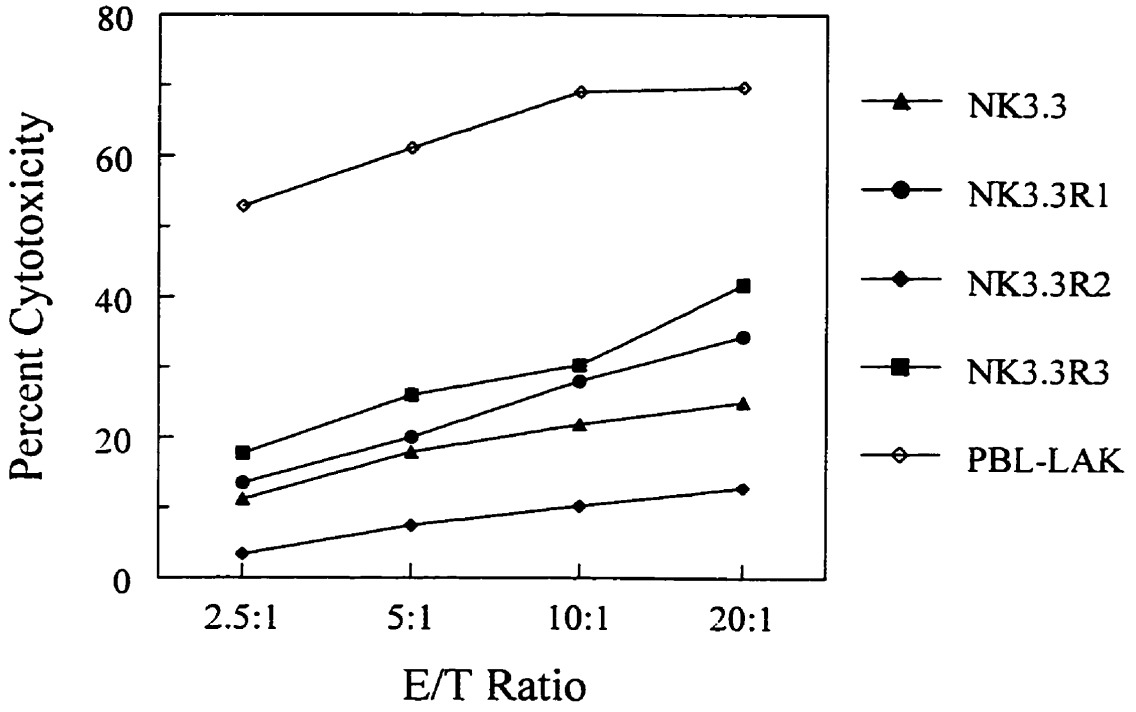
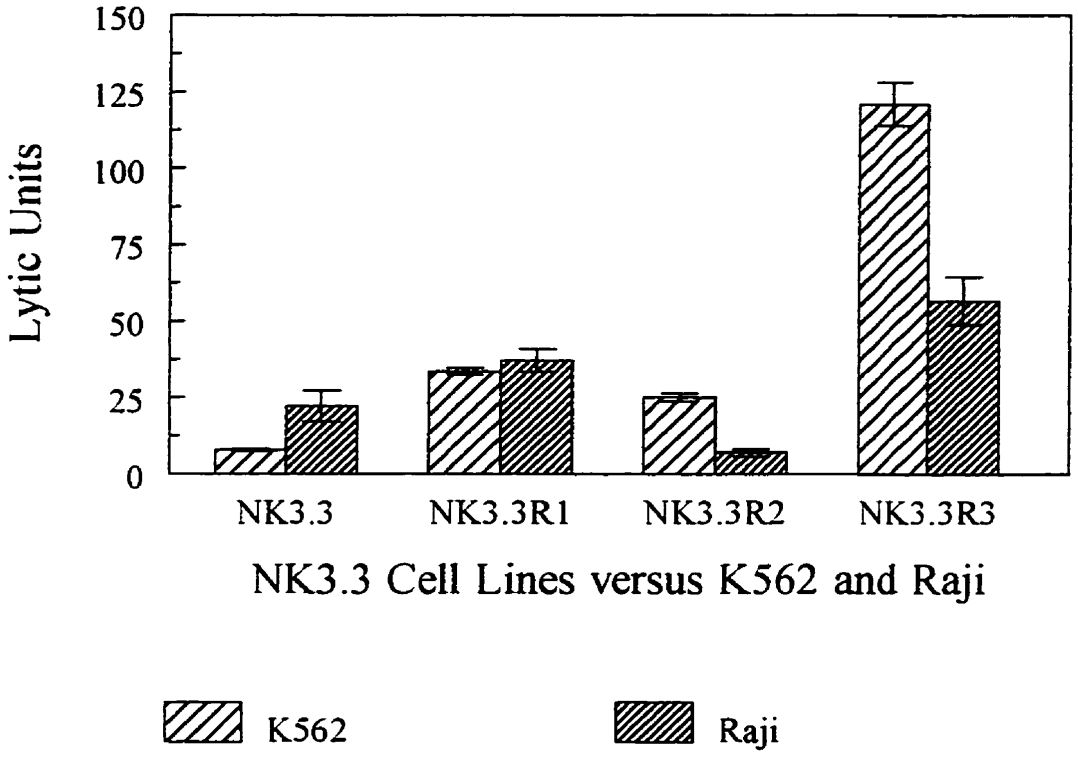
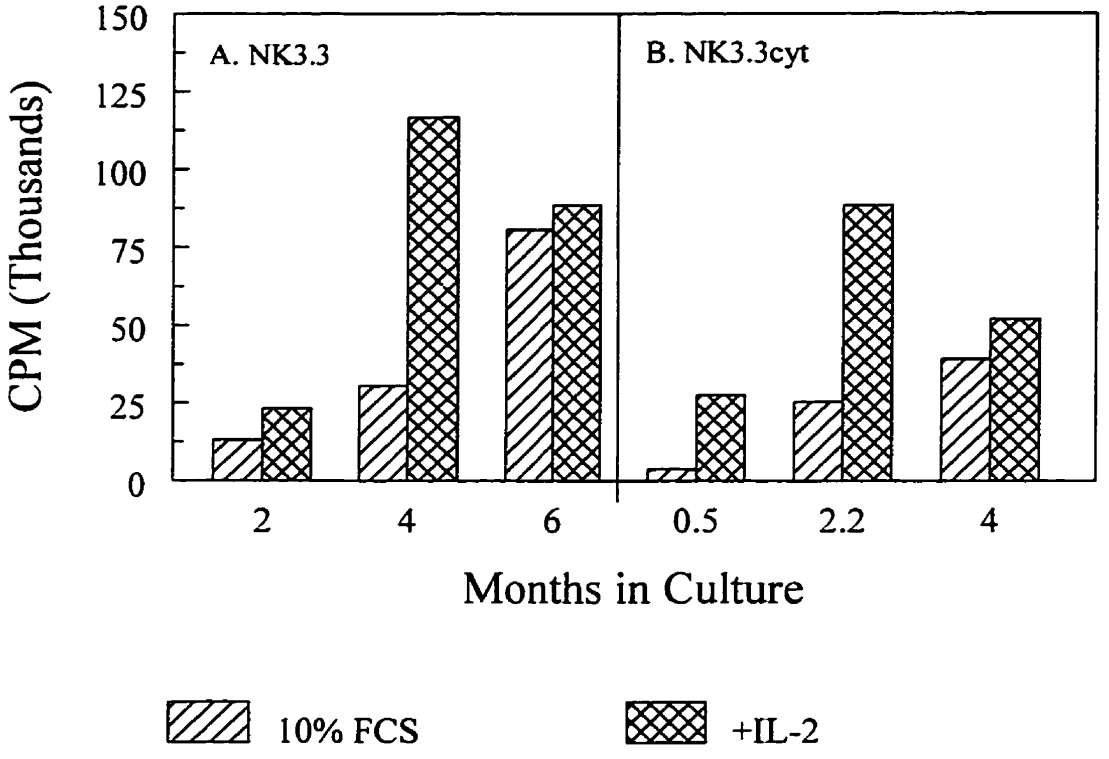

80
60
40
20
0
Percent Cytotoxicity
2.5:1
5:1
10:1
20:1
E/T Ratio
NK3.3
NK3.3R1
NK3.3R2
NK3.3R3
PBL-LAK

Figure 22. LAK activity of NK3.3R1, NK3.3R2 and NK3.3R3 *versus* NK3.3 controls (lytic units). NK3.3 derived cell lines were stimulated with 100 U/ml IL-2 prior to analysis for cytolytic activity in an 18h ^{51}Cr release assay against the NK-resistant and LAK-sensitive target Raji, as described in Materials and Methods. Cytotoxicity is expressed in lytic units. NK3.3 R1 and NK3.3 R3 were significantly more cytotoxic against Raji and K562 than NK3.3 ($p<0.05$). NK3.3 R2 was less cytotoxic against Raji than the NK3.3 control, but more cytotoxic against K562 ($p<0.05$).Significance was determined from non-overlapping 95% confidence intervals.


150
125
100
75
50
25
0
Lytic Units
NK3.3
NK3.3R1
NK3.3R2
NK3.3R3
NK3.3 Cell Lines versus K562 and Raji
K562
Raji

the receptor (Satoh *et al.*, 1991). At the same time, the same cytokines activate a variety of other intracellular mediators, such as the previously mentioned STATs, that do not use the Ras pathway in order to activate gene transcription (Darnell, 1997). The question asked was whether Ras activation on its own is enough to stimulate the proliferation of the cells or will it synergise with the cytokines used in the proliferative response of these cells. The cytokines were chosen based on previous work in the lab showing that there are differences in the response to cytokines between the cytotoxic freshly thawed NK3.3 and a non-cytotoxic variant obtained by long term culture in IL-2 (Majury, 1997). The proliferative response of NK3.3, NK3.3neo, NK3.3R1, R2 and R3 was measured in a 72h tritiated thymidine assay. In each experiment, the controls and the $Ras^{Arg\text{-}12}$ expressing cell lines had been maintained in culture in the presence of IL-2 for the same length of time. In addition, a freshly thawed highly cytotoxic NK3.3 clone was used as a positive control. Figure 23 illustrates the kinetics of the proliferation of the NK3.3 cell line maintained in culture in the presence of IL-2, showing that with time, the proliferation rate of the cells in the presence of serum alone changes. The NK3.3 represents a cell line thawed at the same time as the NK3.3R1, R2 and R3 cell lines, which gradually lost its cytotoxic function. The NK3.3cyt is a cell line which was freshly thawed as a control for NK3.3 and maintained in culture for a much shorter period of time, at three time points in culture. The proliferation rate is represented on the Y axis in cpm of ^{3}H-thymidine incorporated by the cells in a 72h proliferation assay performed as described in Materials and Methods. The results in Figure 23 show that with time in culture the NK3.3 cell line proliferated more in response to serum and, by 4-6 months, became refractory to IL-2, at about the same time that a reduction in the cytotoxic function was observed.

Figure 23. Time-course of the proliferation of NK3.3 in response to IL-2 and to serum. (Comparison between a cytotoxic freshly thawed NK3.3 and non-cytotoxic NK3.3) NK3.3 cells were cultured long term. At the indicated time intervals the cells were set in a 72h tritiated thymidine assay as described in the Materials and Methods. At any given time, a cytotoxic freshly thawed NK3.3 cell line was also used in the assay. Results are presented as counts per minute. IL-2 = Interleukin 2 100 U/ml. Standard deviations of the triplicate cpm per well were less than 1% of the mean and are not shown.


150
125
100
75
50
25
0
CPM (Thousands)
A. NK3.3
B. NK3.3cyt
2
4
6
0.5
2.2
4
Months in Culture
10% FCS
+IL-2

In contrast to the NK3.3 controls, the NK3.3R1, R2 and R3 cell lines were still responsive to IL-2 despite the fact that they had been maintained in culture for the same length of time as NK3.3 (Fig. 24). Differences in the response, expressed as the proliferation index, were seen between the three $Ras^{Arg\text{-}12}$ expressing cell lines and between experiments. Despite these differences NK3.3R1, R2 and R3 all had a higher proliferation index than the controls. However, as with the NK3.3 controls, there was a positive correlation between the proliferation index in response to IL-2 and the cytotoxicity of the Ras expressing sublines against K562 (Fig 25).

Further experiments were done to determine the effect of IL-7 and stem cell factor on the proliferative ability of NK3.3 and the Ras-expressing sublines. The results are shown in Fig. 26 (a single experiment) and Fig. 27 (pooled data from 3 experiments). These cytokines have been chosen because they had been shown to stimulate a heavily passaged, non-cytotoxic variant of NK3.3 in previous experiments in the laboratory (Majury, 1997). In the NK3.3 Ras cell lines, however, no effect of IL-7, SCF or the two combined was observed. In other experiments (data not shown) it was found that IFNγ, at a concentration of 500 U/ml and 1000 U/ml, also had no effect on the cell lines.

In summary, the results shown in this section demonstrate that IL-2 is needed for optimal proliferation of the NK3.3 v-H- Ras expressing cell lines. IFN, SCF, or IL-7/SCF in combination, had no effect on the proliferation of either NK3.3 or the v-Ras transduced NK3.3 sublines. In general a higher proliferation index in response to IL-2 correlated with higher cytotoxicity of the cell lines.

Figure 24. Proliferation of the NK3.3R1, NK3.3R2 and NK3.3R3 *versus* NK3.3 control, in presence or absence of IL-2. The cells were set in a 72h tritiated thymidine assay as described in the Materials and Methods. Results are presented as counts per minute. IL-2 = Interleukin 2 100 U/ml. Standard deviations of the triplicate cpm per well were less than 1% of the mean and are not shown. Proliferation of the three NK3.3 Ras sublines was significantly higher in response to IL2. This was not the case with the long-term cultured NK3.3 control. Significance was determined from non-overlapping 95% confidence intervals.

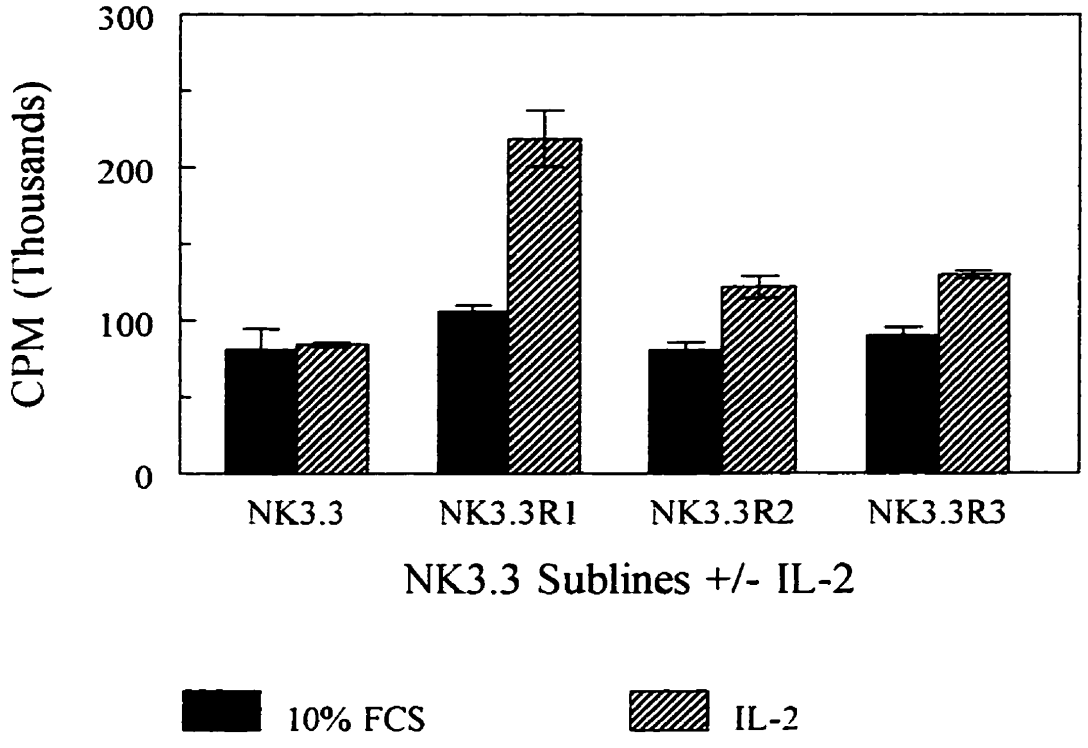

300
200
100
0
CPM (Thousands)
NK3.3
NK3.3R1
NK3.3R2
NK3.3R3
NK3.3 Sublines +/- IL-2
10% FCS
IL-2

Figure 25. Correlation between proliferation in response to IL-2 and cytotoxicity of NK3.3 cell lines. The relationship between the cytotoxic ability of the cells expressed in lytic units and the responsiveness of the cells to IL-2 expressed as proliferation index (PI) are presented for each cell line at 3 time points in culture. PI = cpm(test)/cpm control. Correlation coefficient r=0.802.

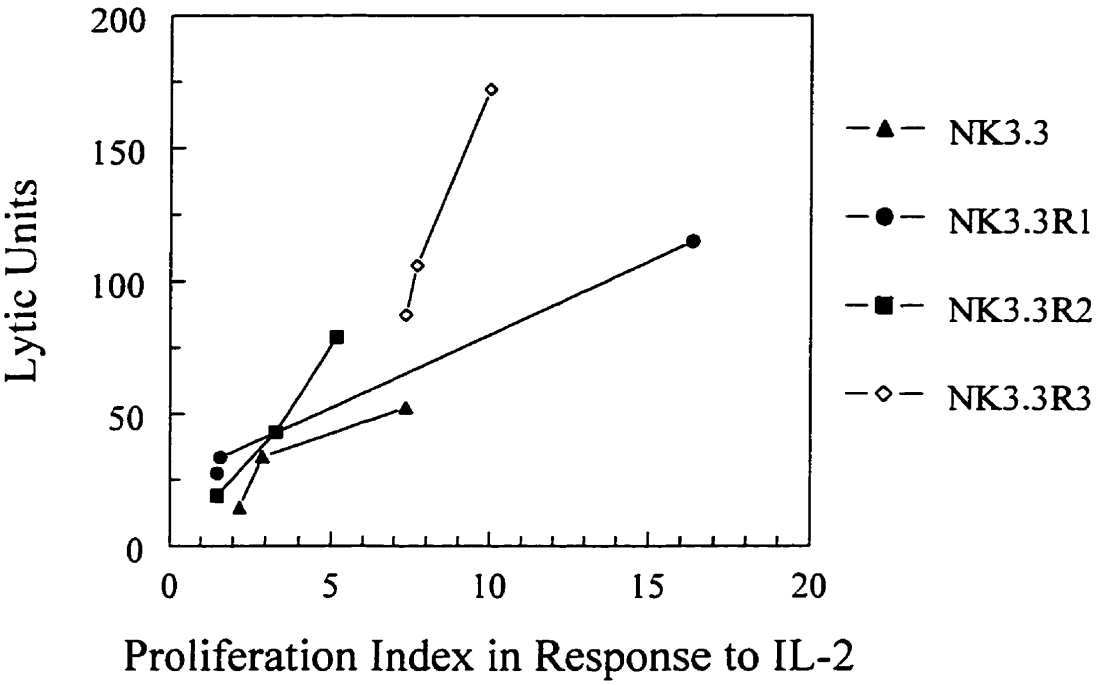

200
150
100
50
0
Lytic Units
0
5
10
15
20
Proliferation Index in Response to IL-2
NK3.3
NK3.3R1
NK3.3R2
NK3.3R3

Figure 26. Comparison of proliferative response of NK3.3R1, NK3.3R2 and NK3.3R3 *versus* NK3.3 control to the cytokines IL-2, SCF and IL-7. The cells were set in a 72h tritiated thymidine assay as described in the Materials and Methods. The results from a representative experiment are reported as proliferation index. IL-2 = Interleukin 2 (100U/ml), SCF = Stem Cell Factor (20ng/ml), IL-7 = Interleukin 7 (10ng/ml).Standard deviations of the triplicate cpm per well were less than 1% of the mean and are not shown. Proliferation of the three NK3.3 Ras sublines was significantly higher than that of NK3.3 in response to IL2 ($p<0.05$), but not in response to the other cytokines. Significance was determined from non-overlapping 95% confidence intervals.

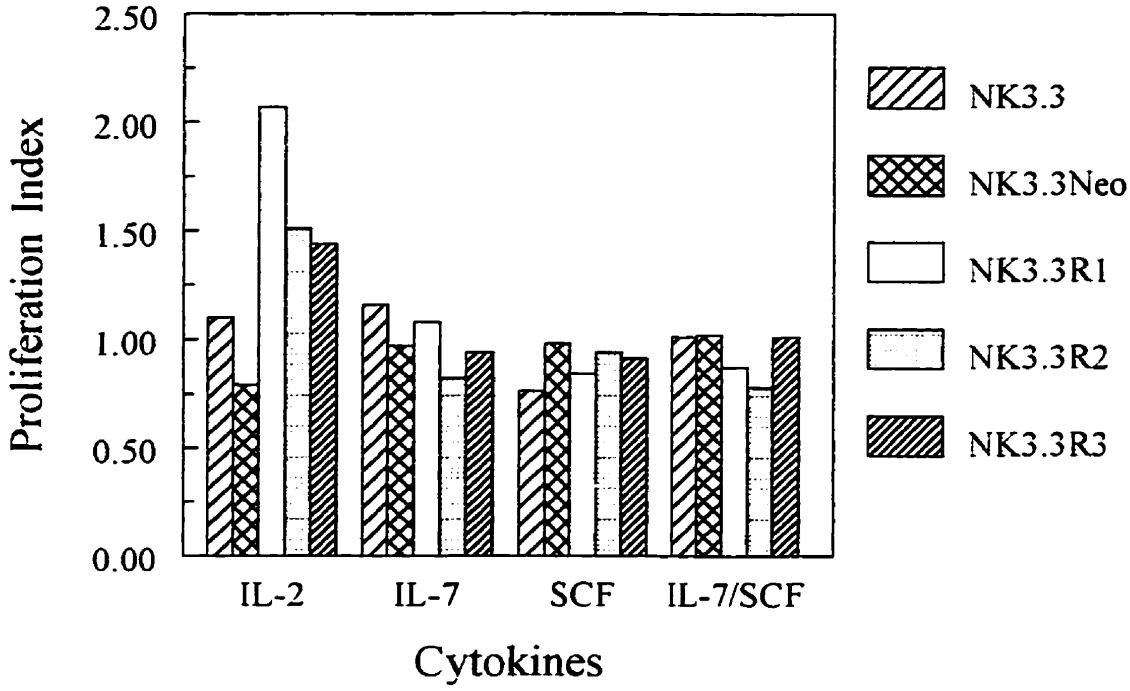
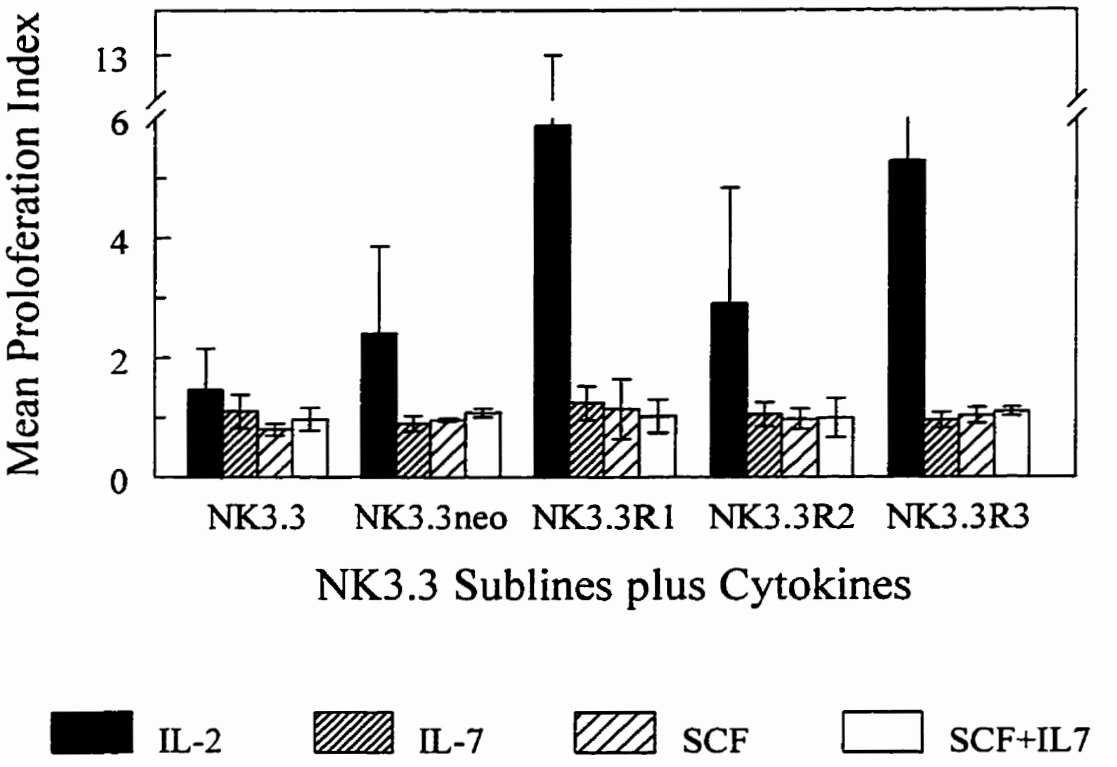

2.50
2.00
1.50
1.00
0.50
0.00
Proliferation Index
IL-2
IL-7
SCF
IL-7/SCF
Cytokines
NK3.3
NK3.3Neo
NK3.3R1
NK3.3R2
NK3.3R3

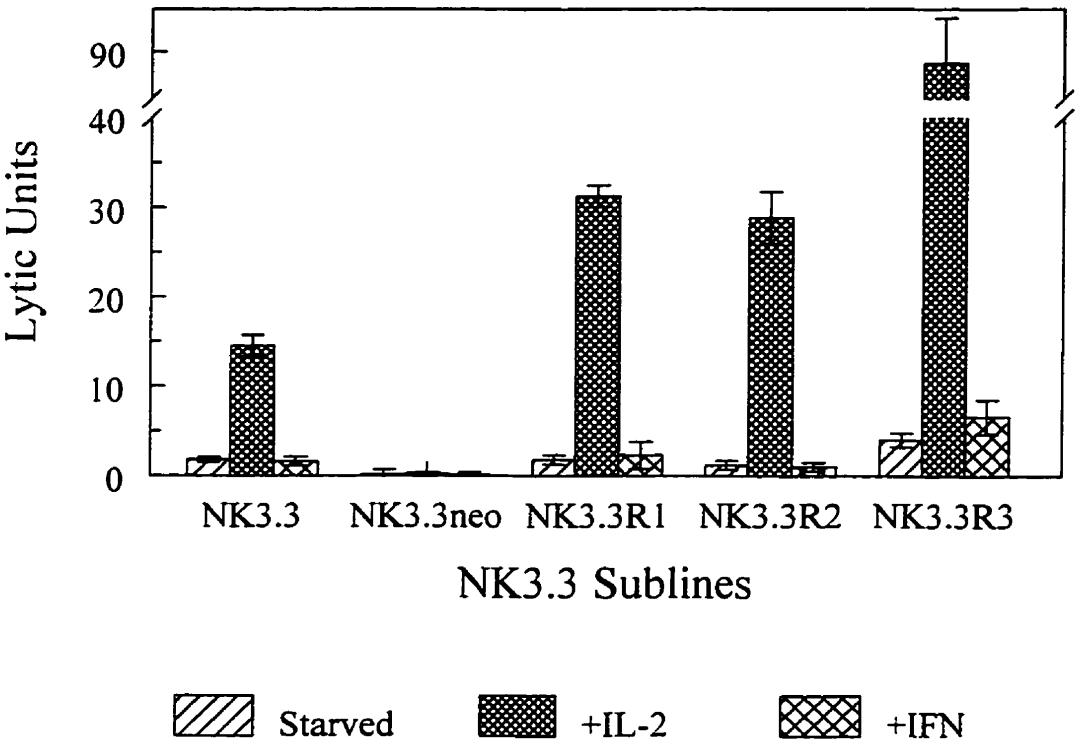
Figure 27. Proliferation of the NK3.3 derived cell lines in response to IL-2, IL-7 and SCF (pooled data). The cells were set in a 72h tritiated thymidine assay as described in the Materials and Methods. The graph represents the means from three experiments +/- S.D. Results are presented as proliferation index. IL-2 = Interleukin 2 (100U/ml), SCF = Stem Cell Factor (20 ng/ml), IL-7 = Interleukin 7 (10 ng/ml). Proliferation of the NK3.3 Ras sublines was significantly greater than that of the NK3.3 control in response to IL2. IL-2 induced proliferation of NK3.3 R1 and NK3.3 R3 were also significantly greater than that of the NK3.3 neo control ($p<0.05$). There was no significant proliferative response to IL7, SCF or IL7/SCF by any of the lines.


Mean Proloferation Index
13
6
4
2
0
NK3.3
NK3.3neo
NK3.3R1
NK3.3R2
NK3.3R3
NK3.3 Sublines plus Cytokines
IL-2
IL-7
SCF
SCF+IL7

The Effect of Exogenous IL-2 and IFNγ on the Cytotoxic Function of NK3.3 Sublines

Based on the results shown above, it was decided to investigate whether IL-2 is needed for the optimal cytotoxic function of the NK3.3 cell lines expressing the activated mutant of Ras, or whether constitutive activation is enough to confer a higher cytotoxic ability. For this purpose the cells were first starved of IL-2 and then stimulated with 500 U/ml for 24h prior to the ^{51}Cr release assay. In addition, the effect of IFNγ was investigated. It has been shown that IFN increases the cytotoxicity of NK cells from peripheral blood (Argov and Klein, 1983). Similar to cytokine receptors, interferon receptors do not have a catalytic domain, and recruit to the membrane members of the STAT family of proteins which, upon stimulation by phosphorylation on tyrosine, form complexes which translocate to the nucleus, and activate gene expression (Gutch *et al.*, 1992; Schindler *et al.*, 1992; Silvennoinen *et al.*, 1993). Since this signal transduction pathway is independent of Ras it was hypothesized that treatment of the cells with IFN would be additive to the effect of Ras activation. For this purpose the NK3.3 sublines were starved of cytokines and then stimulated with IFNγ and with IL-2 as a positive control, followed by an 18h ^{51}Cr release assay. The results of one such experiment are presented in Figure 28. IFNγ had little effect on the cytotoxicity of the NK3.3 control or the NK3.3R1, R2 or R3. However, treatment of the cells with IL-2 resulted in increased cytotoxicity of all NK3.3 sublines. Differences exist between the NK3.3 control and the Ras expressing sublines, NK3.3R1, R2 and R3 exhibiting higher cytotoxicity than the control NK3.3, with NK3.3R3 consistently

Figure 28. The effect of IFNγ and IL-2 on cytotoxic function of NK3.3, NK3.3R1, NK3.3R2 and NK3.3R3. NK3.3 cells were starved of IL-2 for 24 hours, followed by stimulation with 1000 U/ml IFN, or 500 U/ml IL-2 for 24 hours prior to the assessement of the cytotoxic function of the cells in a ^{51}Cr release assay. The results are presented in lytic units. The increase in cytotoxicity was significant in response to IL-2 only. Significance was determined from non-overlapping 95% confidence intervals.


Lytic Units
90
40
30
20
10
0
NK3.3
NK3.3neo
NK3.3R1
NK3.3R2
NK3.3R3
NK3.3 Sublines
Starved
+IL-2
+IFN

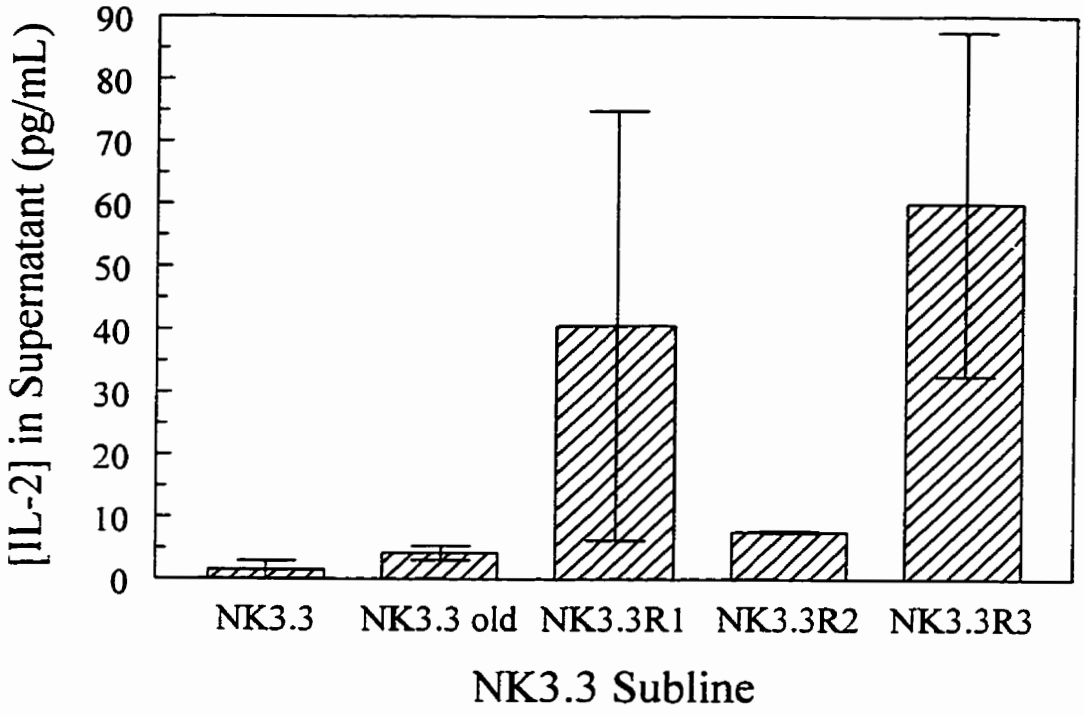
higher than the others. It can also be seen that Ras activation alone is not capable of making the cells cytotoxic since the Ras^{Arg-12} expressing cell lines, when starved of IL-2, are not capable of killing the K562 cell line more effectively than the non v-Ras expressing NK3.3

Cytokine Production by NK3.3 Sublines.

$p21^{ras}$ has been linked to the activation of the transcription factors of the NFAT (nuclear factor of activated T cells) family, which control the transcriptional activation of cytokine genes such as IL-2, IL-4 or GM-CSF (Liu *et al.*, 1997). As previously described, NK3.3 which have been maintained long term in culture, became IL-2 independent, and might be autocrine for IL-2. Furthermore, stimulation of NK cells with IL-2, which activates Ras, leads to increased secretion of TNFa and IFNγ. Based on the observation that IL-2 and CD16 activate not only Ras, but other signalling proteins as well e.g., PI-3 kinase, PKC, JNK, etc. (Azzoni *et al.*, 1992; Ting *et al.*, 1992; Park *et al.*, 1993), the question asked was whether or not Ras activation alone is sufficient for the increased secretion of IL-2, TNF and IFN by NK3.3 cells. In order to assess the IL-2 production of the NK3.3 cell lines, $1x10^6$ cells/ml were incubated in RPMI 10% FCS for 72h, the supernatants were collected, and IL-2 concentrations were measured by ELISA as described in Materials and Methods. The mean of two experiments is shown in Figure 29.

It can be seen from this figure that the NK3.3 Ras cell lines secrete significantly larger amounts of IL-2 than the controls ($p<0.05$). Interestingly, NK3.3R3 produces the highest levels of IL-2 (Fig. 29). A slight increase in IL-2 production can also be seen in the NK3.3 cell line which has been maintained longer in culture, although to a much

Figure 29. IL-2 production of NK3.3, NK3.3R1, NK3.3R2 and NK3.3R3. The NK3.3 cell lines were cultured in culture medium without IL-2 for 72 hours. Supernatants were collected and IL-2 concentration was determined by ELISA as described in Materials and Methods. The figure shows the mean of two experiments, ± S.D. Statistics were not done because of the small sample size and large standard deviation.


[IL-2] in Supernatant (pg/mL)
90
80
70
60
50
40
30
20
10
0
NK3.3
NK3.3 old
NK3.3R1
NK3.3R2
NK3.3R3
NK3.3 Subline

lesser extent than the NK3.3 sublines expressing Ras^{Arg-12}.

TNF and IFN production by the NK3.3 cell lines was assessed as follows. For the first two experiments, the NK3.3 cell lines were plated at a concentration of 2×10^5 cells/ml in RPMI in 10% FCS without the addition of IL-2 for 24 hours, in order to avoid the contribution of IL-2 to the activation of Ras in the controls. The supernatants were then collected and IFN or TNF concentrations were measured by ELISA as described in the Materials and Methods. Figures 30 and 31 show the results from two experiments performed as described above. IFN production after 24h incubation in the absence of IL-2 (Fig. 30), was lower in the v-Ras expressing cell lines than in the control. The same pattern can be seen in Figure 31 showing the TNF production under the same conditions, indicating that the NK3.3R1, NK3.3R2 and NK3.3R3 secrete less TNF than their NK3.3 controls, although the overall amount secreted is very low. A third set of experiments (Figs. 32 and 33) was then carried out in which the cells were maintained in culture for 24h under the same conditions as above, and then stimulated with IL-2 for 48h or left with RPMI with 10% FCS. The supernatants where then collected and IFN or TNF concentrations were measured. Figure 32 shows the IFN production by NK3.3 derived cell lines. The first column represents the concentration of IFN in the supernatants after 72h incubation with RPMI 10% FCS only. The second column represents IFN production following IL-2 stimulation of the cells for 48h. The overall amounts of IFN are higher in this experiment due to longer incubation in culture, and all cell lines secreted higher amounts of IFN (approximately two-fold) following IL-2 stimulation. Although some variability exists, no significant differences were seen between the cell lines expressing Ras^{Arg-12} and the controls. TNF production by the NK3.3 cell lines from a 72h assay is

Figure 30. Production of IFNγ by NK3.3 cell lines. The NK3.3 cell lines were cultured in culture medium without IL-2 for 24 hours. Supernatants were collected and IFNγ concentration was determined by ELISA, as described in Materials and Methods. The results from two separate experiments are presented in the figure. Statistics were not done because of the small sample size.

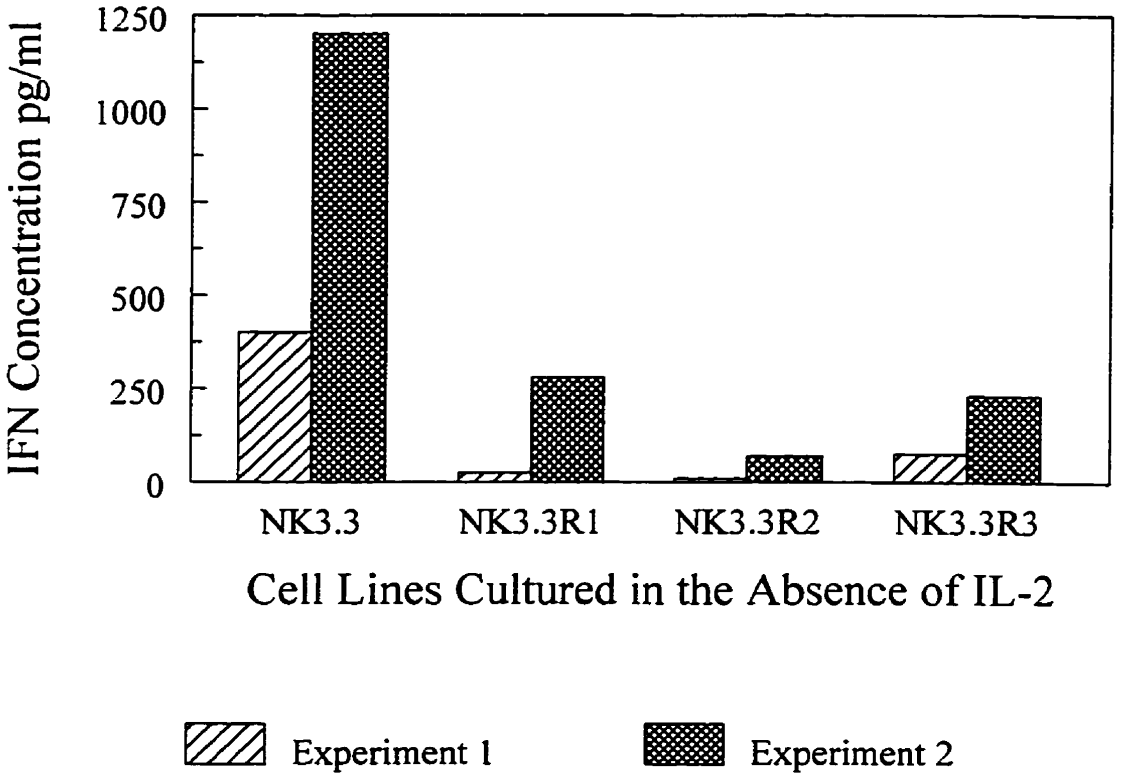

IFN Concentration pg/ml
1250
1000
750
500
250
0
NK3.3
NK3.3R1
NK3.3R2
NK3.3R3
Cell Lines Cultured in the Absence of IL-2
Experiment 1
Experiment 2

Figure 31. Production of TNFα by the NK3.3 cell lines. The NK3.3 cell lines were cultured in culture medium without IL-2 for 24 hours. Supernatants were collected and TNFα concentration was determined by ELISA, as described in Materials and Methods. The results from two separate experiments are presented in the figure. Statistics were not done because of the small sample size.

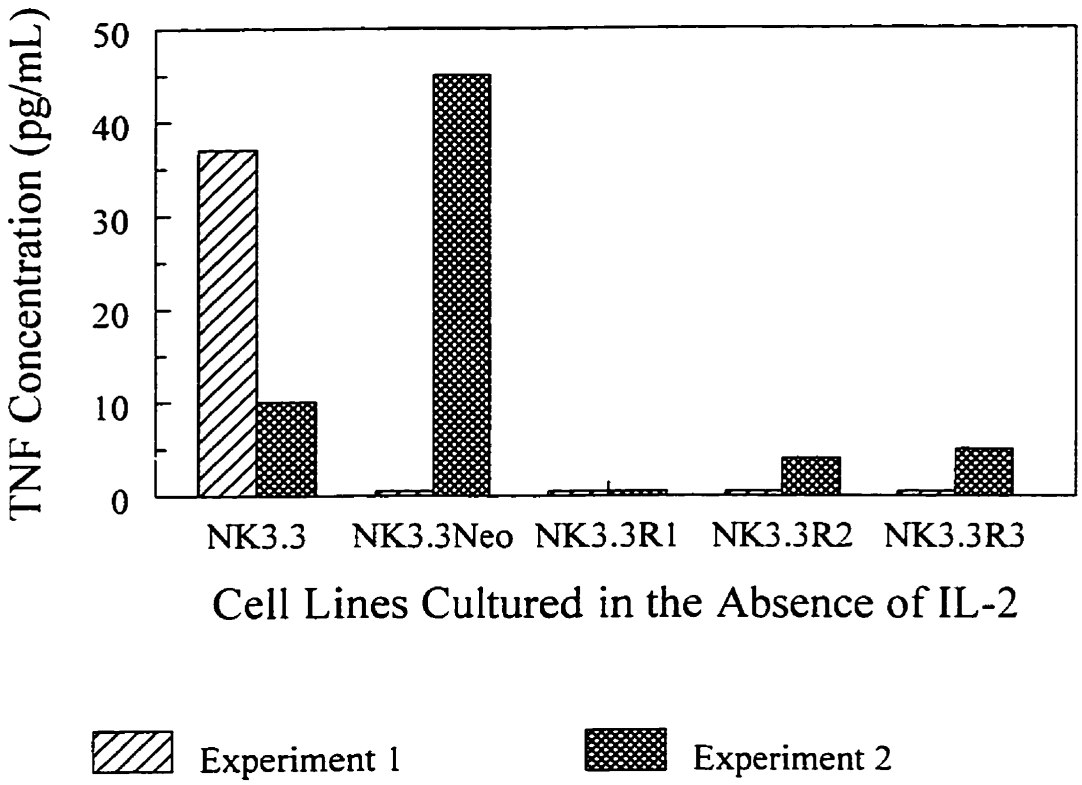
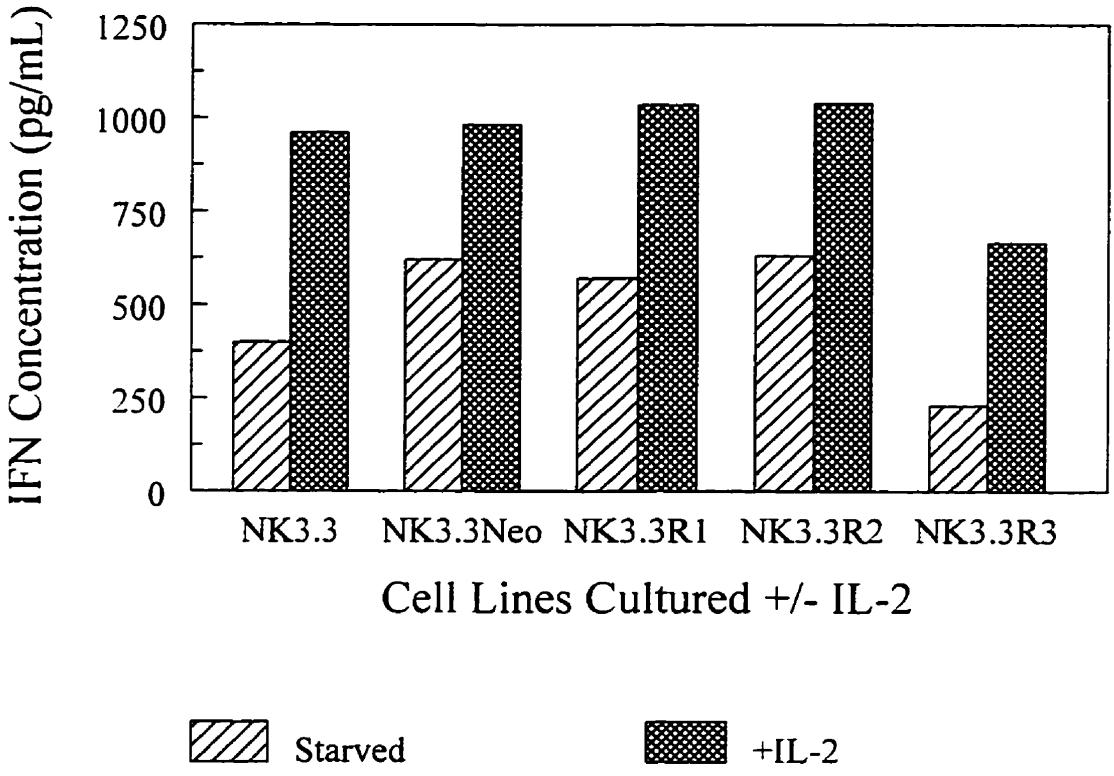

TNF Concentration (pg/mL)
50
40
30
20
10
0
NK3.3
NK3.3Neo
NK3.3R1
NK3.3R2
NK3.3R3
Cell Lines Cultured in the Absence of IL-2
Experiment 1
Experiment 2

Figure 32. IL-2 stimulation increases IFNγ production of NK3.3 cell lines. The NK3.3 cell lines were cultured in IL-2 free culture medium for 24 hours, prior to stimulation with 500 U/ml IL-2 for 48h or continued to be cultured in IL-2 free medium. Supernatants were collected and IFNγ concentration was determined by ELISA, as described in Materials and Methods. Statistics were not done because of the small sample size.

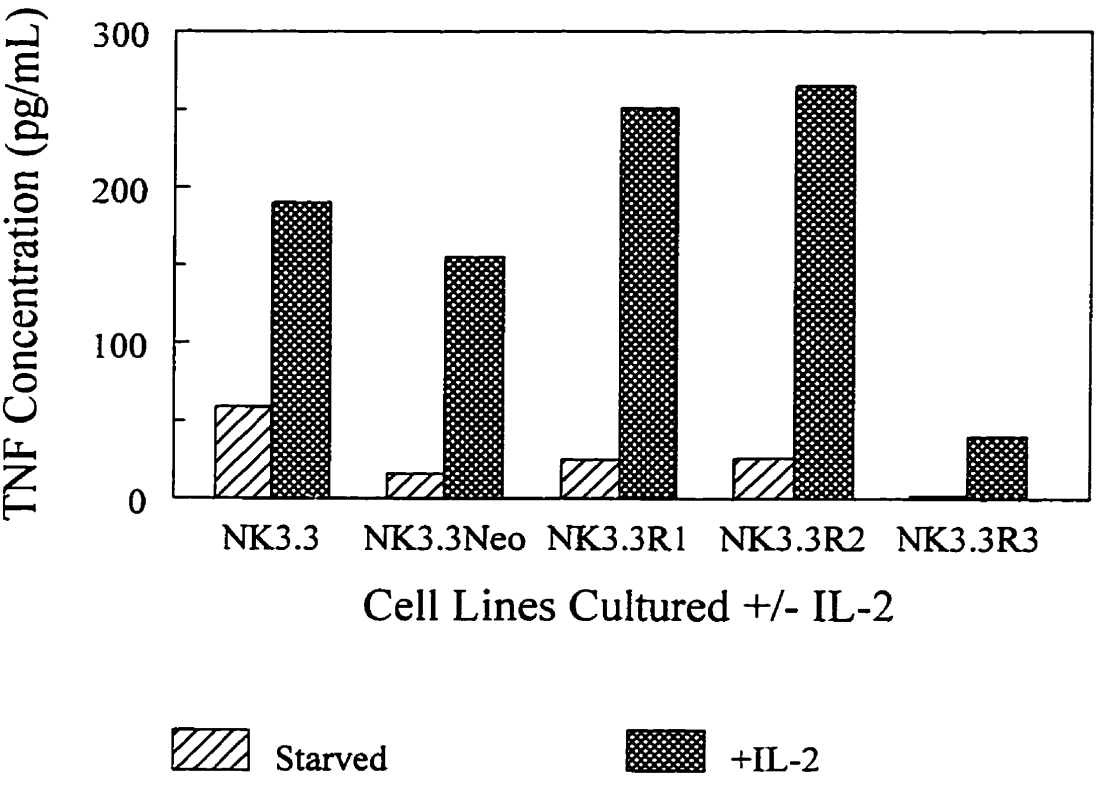

IFN Concentration (pg/mL)
1250
1000
750
500
250
0
NK3.3
NK3.3Neo
NK3.3R1
NK3.3R2
NK3.3R3
Cell Lines Cultured +/- IL-2
Starved
+IL-2

presented in Figure 33. TNF production without IL-2 stimulation was low in all NK3.3 cell lines. Stimulation with IL-2 increased TNF production by approximately 10-fold in all NK3.3 sublines. NK3.3R1 and NK3.3R2 secrete the highest amounts of TNF when stimulated with IL-2, but in this experiment NK3.3R3 seemed to secrete the lowest amount.

More experiments could have been done in order to confirm the differences between the cell lines, but unfortunately this was not possible due to the high cost of the reagents needed for the ELISA.

In summary, the results shown in this section show that activation of Ras is sufficient for the increased production of IL-2 in NK3.3. However, no differences in TNF or IFN production could be seen between the $Ras^{Arg\text{-}12}$ expressing NK3.3 cell line and the controls.

Figure 33. IL-2 stimulation increases TNFα production of NK3.3 cell lines. The NK3.3 cell lines were cultured in IL-2 free culture medium for 24 hours, prior to stimulation with 500 u/ml IL-2 for 48h or continued to be cultured in IL-2 free medium. Supernatants were collected and IFNγ concentration was determined by ELISA, as described in Materials and Methods. Statistics were not done because of the small sample size.


TNF Concentration (pg/mL)
300
200
100
0
NK3.3
NK3.3Neo
NK3.3R1
NK3.3R2
NK3.3R3
Cell Lines Cultured +/- IL-2
Starved
+IL-2

Conjugate Formation by NK3.3 NK3.3R1, R2 and NK3.3R3 to the K562 Cell Line

One of the first steps in the cytolytic process mediated by NK cells is target cell binding. Due to the differences in the cytotoxicity of the NK3.3 cell lines that express the activated form of Ras, it was decided to investigate whether differences exist also in the conjugate formation frequency of the four sublines. These experiments were performed by a two colour flow cytometry assay primarily by S. C. Juvet, under my supervision, as described in Materials and Methods. Figure 34 shows the percentage of conjugates formed by each of the cell lines in five independent experiments. The differences between the sublines were found to be statistically different in a two way ANOVA test ($p<0.05$). The cell line NK3.3R3, which is the most cytotoxic, formed fewer conjugates with the tumour target K562 as compared to the parental NK3.3 clone which formed the highest number of conjugates. Figure 35 shows the mean percentage of NK3.3 sublines in conjugates for the five experiments shown in Figure 34. A two tailed paired t-test comparing the NK3.3 clone to the Ras^{Arg-12} expressing sublines showed that the frequency of conjugates formed by NK3.3R1 ($p<0.05$) and NK3.3R3 ($p<0.05$) was significantly lower than the parental NK3.3 clone. The frequency of conjugate formation by NK3.3R2 was not significantly different from the control, although an apparent difference could also be seen. Figure 36 shows the correlation between the cytotoxic function of the NK3.3 sublines expressed as the mean of two ^{51}Cr release assays performed prior and following the conjugate formation assay *versus* the mean percentage of conjugates formed by the NK3.3 sublines. An inverse correlation was found between the cytotoxic ability of the cell lines and the conjugate formation frequency.

Figure 34. Conjugate formation between NK3.3 cell lines and K562. NK3.3 cell lines were stained with calcein and K562 cells were stained with hydroethidine, as described in Materials and Methods. 2 x 10^5 effector cells were mixed with 2 x 10^5 K562 cells, pelleted by centrifugation at 200 x g and incubated at room temperature for 10 min. prior to analysis by flow cytometry. Conjugates were determined as a double positive event. The results from five separate experiments are presented in the figure.

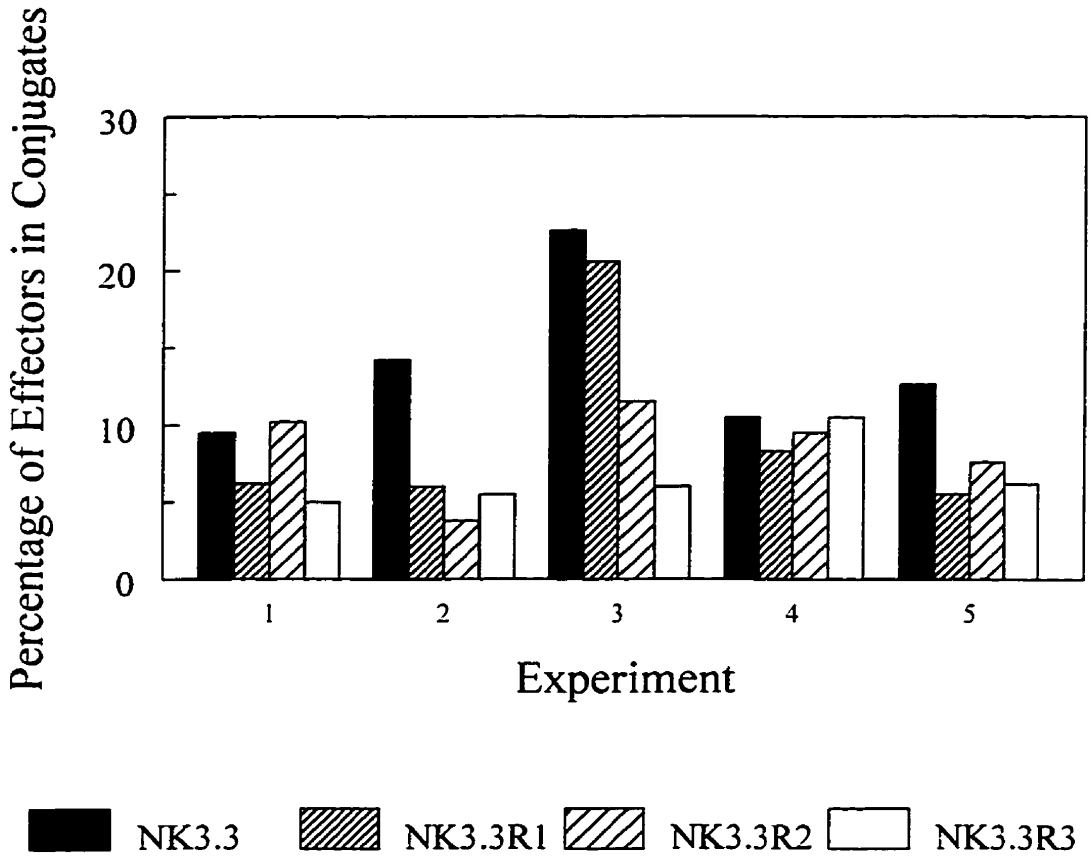

Percentage of Effectors in Conjugates
30
20
10
0
1
2
3
4
5
Experiment
NK3.3
NK3.3R1
NK3.3R2
NK3.3R3

Figure 35. Evaluation of NK3.3, NK3.3R1, NK3.3R2 and NK3.3R3 binding to the tumour target K562. The cell lines were stained and handled as described in Fig. 34. The mean percentages for the experiments presented in Figure 35 are presented, error bars represent the standard error.

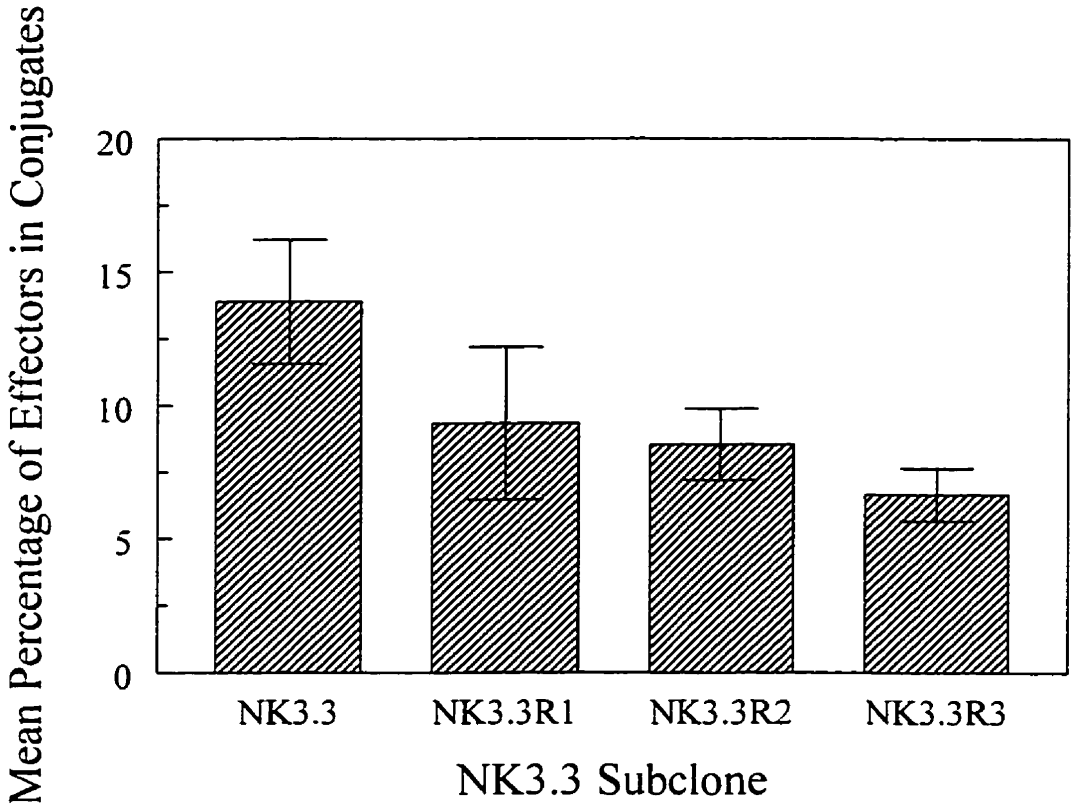
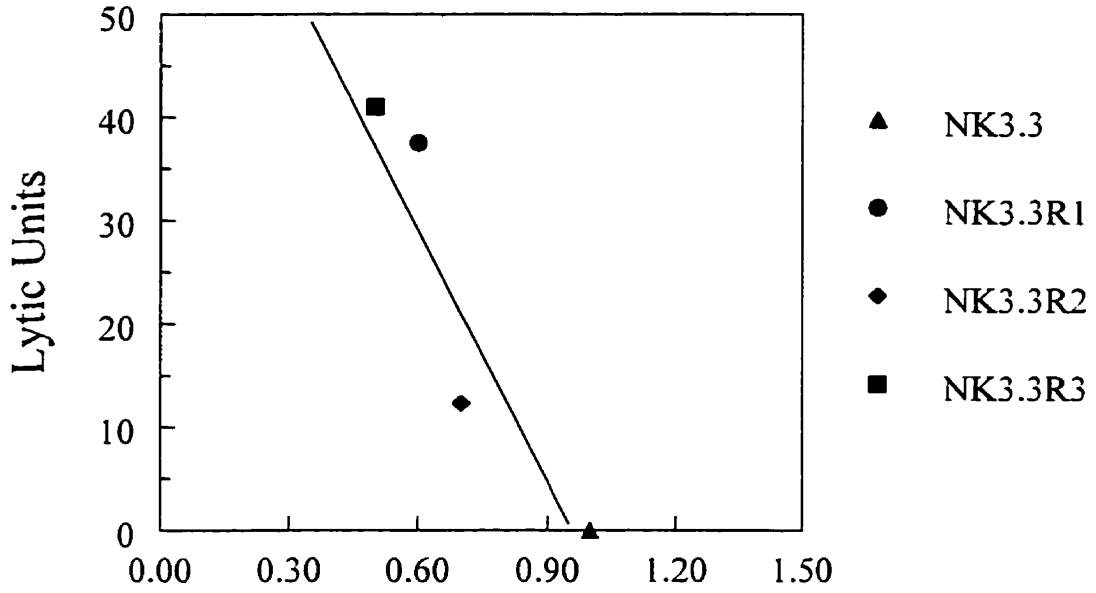

Mean Percentage of Effectors in Conjugates
20
15
10
5
0
NK3.3
NK3.3R1
NK3.3R2
NK3.3R3
NK3.3 Subclone

Figure 36. Inverse correlation between cytotoxicity (LU(20%)/10^6) and the mean percentages of effectors in conjugates. The cytotoxicity data presented in the Figure are the results of two ^{51}Cr release assays, one performed prior the flow cytometry experiment and the second performed after their completion. The mean percentages of effectors in conjugates were obtained from the experiments presented in Figure 34. The correlation coefficient for the data: r = -0.931.



Mean Frequency of Effectors in Conjugates Relative to Control (NK3.3)

Flow Cytometric Analysis for Antigen Expression of NK3.3 Sublines

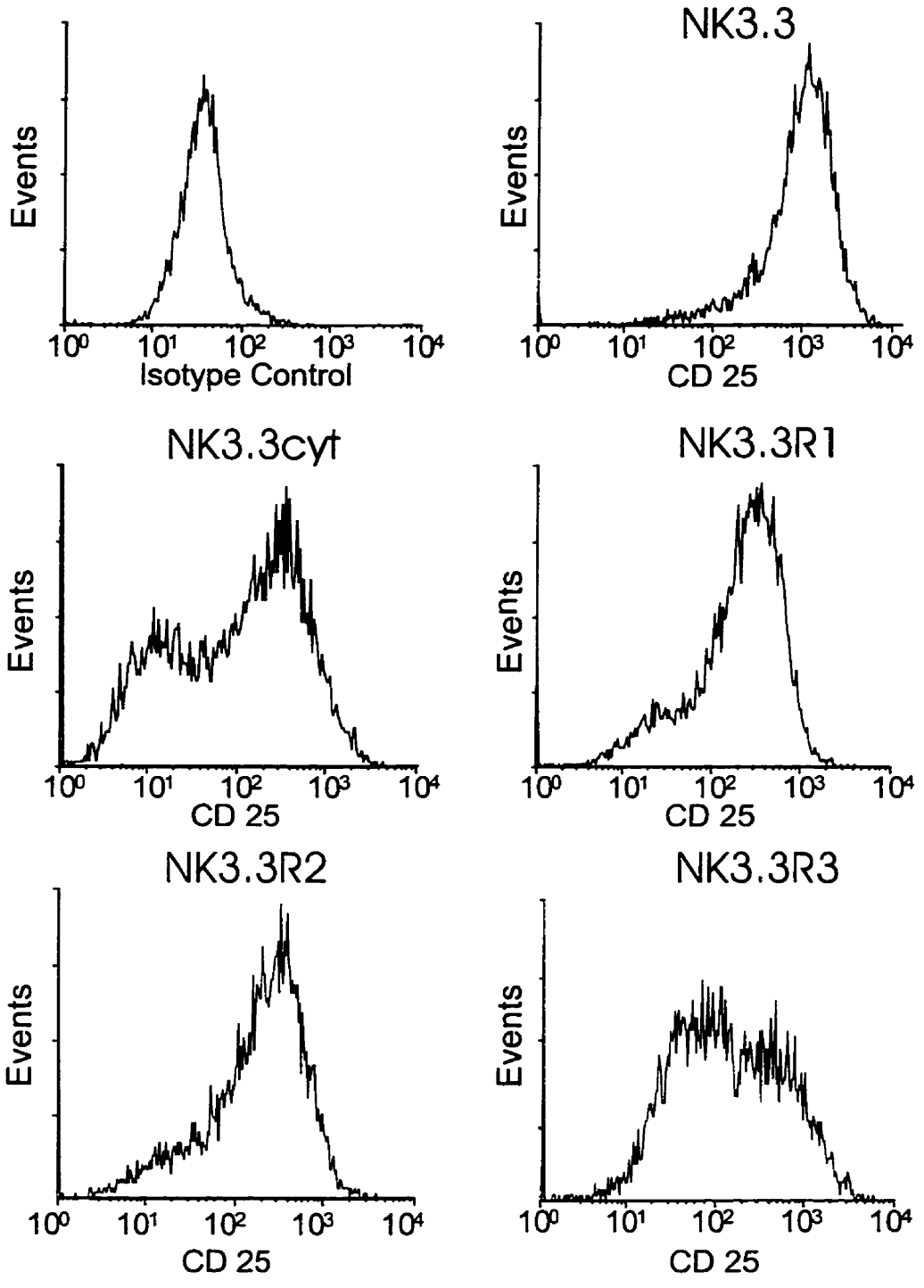
NK cells express CD16, CD56, the IL-2 receptor (CD25) and a number of adhesion molecules such as CD2, CD11b, CD18, CD44, CD54, CD56, CD58, CD69, VLA1, VLA3, VLA4-6. (Gismondi *et al.*, 1992; Palmieri *et al.*, 1995; Gismondi *et al.*, 1991; Chong *et al.*, 1994; Bell *et al.*, 1992; Tan *et al.*, 1993; Muto *et al.*, 1993; Ross and Vetvicka, 1993; Trinchieri, 1989). The expression of some of these molecules changes with NK activation by IL-2 or other means. It has also been shown that treatment of NK cells with TPA or cross-linking of CD16, which activates Ras, increases NK adhesion to laminin via VLA-6, and also that activated Ras and Src induce CD44 expression (Jamal *et al.*, 1994; Gismondi *et al.*, 1992). Furthermore, because a difference in conjugate formation frequency between the NK3.3 cell lines was observed, differences in the expression of adhesion molecules could also be expected. The effect of Ras activation on expression of CD16, CD56, CD25, transferrin receptor and VLA4-6 was investigated.

Table 1 summarizes the results from three independent experiments, which were done in order to investigate the expression of these cellular antigens by flow cytometry. The table shows that expression of $Ras^{Arg\text{-}12}$ increases the percent of positive cells expressing CD56, and CD16. However, the expression of CD25 was increased in the NK3.3 control, when a hundred percent of the cells become positive for the antigen expression, but not in the NK3.3R3 and to a lesser extent in NK3.3R1 and R2, or the cytotoxic, freshly thawed NK3.3. Figure 37 shows the pattern of CD25 expression in the cell lines. The figure shows that a double population of bright and dim expressors for CD25 is present in the NK3.3 cell lines expressing $Ras^{Arg\text{-}12}$, and the NK3.3 cytotoxic cell line but not in the NK3.3 maintained for the same amount of time in culture as the Ras

Table 1. Flow cytometric analysis of the NK3.3 cell lines for the antigens CD16, CD56, CD71, CD25 and CD49d, e, and f. The values presented in the table are the means of three experiments +/- S.D.

Cell Lines		Antigens					
		CD56	CD16	CD25	CD49d	CD49e	CD49f
NK3.3	%pos	0.34	1.48	99.95±0.07 (bright)	99.97 ±0.06	3.92 ±2.14	99.80 ±0.14
	MFI			37.82±43.24 bright)	22.69 ±18.50	3.92 ±2.14	4.13 ±2.88
NK3.3 R1	%pos	6.75	1.09	22.40±11.46 (dim) 77.10±11.46 (bright)	100	99.86 ±0.23	99.70 ±0.14
	MFI			1.85±0.58 (dim) 23.30±6.93 (bright)	6.91 ±3.21	2.19 ±0.74	1.70 ±0.23
NK3.3 R2	%pos	2.41	2.42	18.00±2.26 (dim) 81.70±2.12 (bright)	100	99.96 ±0.06	94.70 ±7.21
	MFI			1.58±0.85 (dim) 21.20±7.21 (bright)	7.03 ±4.61	2.43 ±1.81	1.50 ±0.57
NK3.3 R3	%pos	9.10	11.85	41.95±26.80 (dim) 57.70±27.45 (bright)	100	99.93 ±0.06	95.97±3.90 (dim) 5.80±3.11 (bright)
	MFI			3.31±2.83 (dim) 34.70±25.88 (bright)	7.96 ±5.29	4.12 ±4.10	2.91±1.26 (dim) 36.90±19.80 (bright)

Figure 37. Flow cytometric analysis of CD25 expression of NK3.3, NK3.3R1, R2 and R3. Cells were washed and labelled with FITC conjugated CD25 as described in the Materials and Methods. 10^4 events were acquired for each sample.


Events
10^0 10^1 10^2 10^3 10^4
Isotype Control
NK3.3
CD 25
NK3.3cyt
NK3.3R1
NK3.3R2
NK3.3R3

expressing cell lines. No difference in the expression of CD49d and e were observed, however, a bright population expressing Cd49f is observed in NK3.3R3.

The results presented in the preceding section show that the expression of Ras^{Arg-12} in the NK3.3 cell line modulates the expression of several antigens, *i.e.*, maintains a subpopulation of dim CD25 expressors, increases the percent positive CD16 and CD56 positive cells, and in NK3.3R3, increases the expression of CD49f.

Granule Exocytosis of NK3.3, NK3.3R1, NK3.3R2 and NK3.3R3

Following the binding of the NK cell to its target a series of intracellular changes occur leading to the exocytosis of the intracellular granules. Exocytosis is triggered in NK cells following binding to the target or by crosslinking of the CD16 receptor in ADCC. It has been reported that inhibition of ERK function, which is believed to be downstream of Ras in most cellular responses, inhibits granule exocytosis mediated by CD16 crosslinking in peripheral blood NK cells (Raspadori *et al.*, 1993). Therefore, the total intracellular serine protease activity and the serine protease exocytosis in the NK3.3 sublines were measured in an assay that measured serine protease activity using the synthetic substrate *N*-benzyloxycarboxy-L-lysine thiobenzyl ester (BLT) as described in Materials and Methods. For this purpose NK3.3, NK3.3R1, R2, and R3 cells were incubated with K562 cells or with media as the background measure. The supernatants were then collected and the BLT-esterase activity was measured in a colourimetric assay. The mean of three such experiments are shown in Figure 38. Results are expressed as percentage release after subtracting the spontaneous release of cells incubated with RPMI. No differences were found in the percent of BLT-esterase exocytosis between the control NK3.3 cell line and

Figure 38. BLT-esterase activity in NK3.3 cell lines induced by culture with K562 tumour cells. 5 x 10^6 effector cells were cultured with 2 x 10^7 K562 cells for 4h at 37° C. The supernatants were collected and BLT-esterase activity was measured as described under Materials and Methods. The results are presented as the mean O.D.(450nm) from three independent experiments. Error bars represent the S.D. The differences between NK3.3 R1 and NK3.3 R2 and the NK3.3 control approached, but did not reach, significance ($p<0.08$ and $p<0.07$ respectively). NK3.3 Ras3 was significantly higher than the NK3.3 control ($p<0.05$).

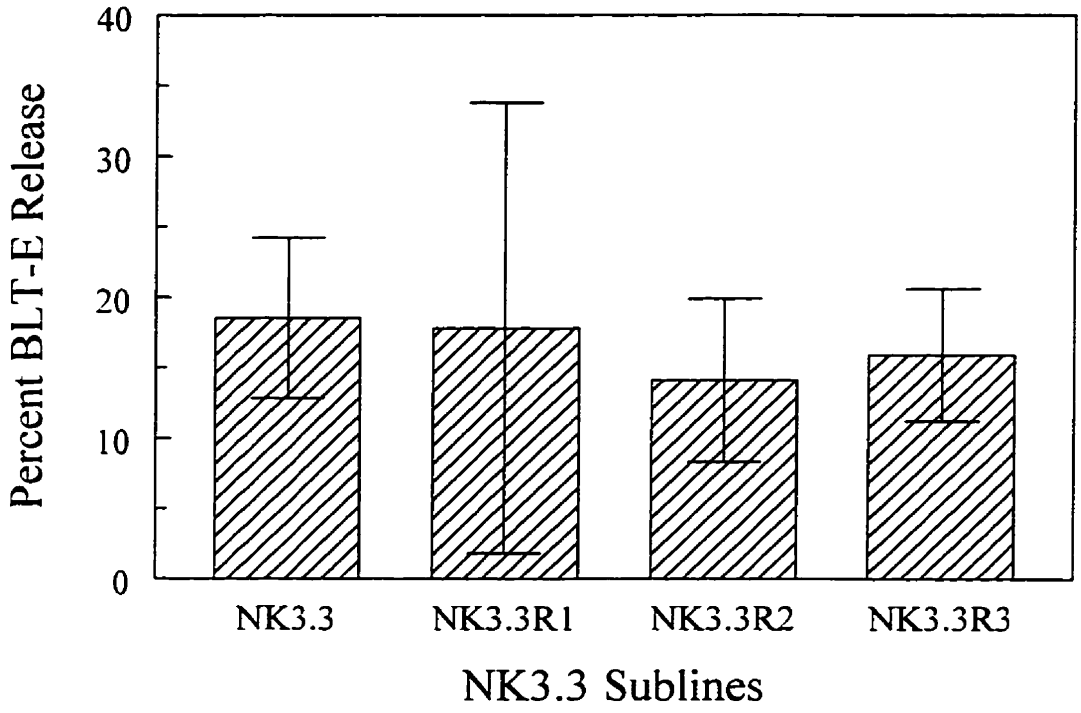
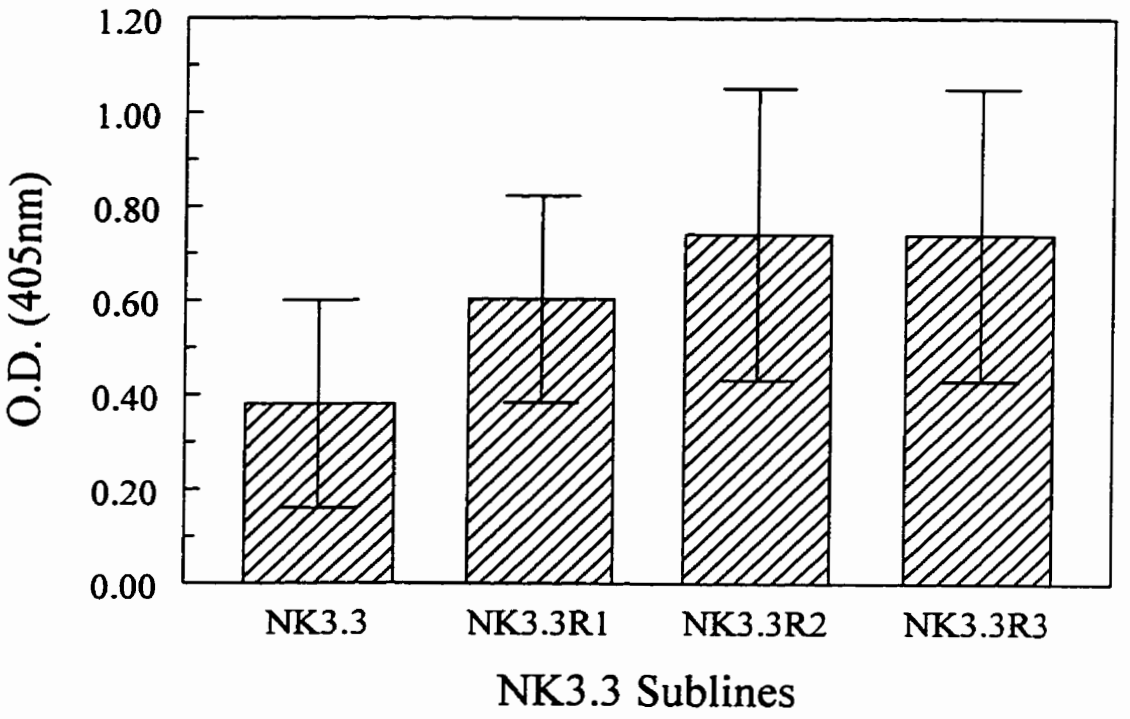

40
30
20
10
0
Percent BLT-E Release
NK3.3
NK3.3R1
NK3.3R2
NK3.3R3
NK3.3 Sublines

Figure 39. BLT-esterase activity in cell lysates of NK3.3, NK3.3R1, NK3.3R2 and NK3.3R3. 5 x 10^6 cells were lysed in PBS-1% NP-40. The BLT-esterase activity was determined as described under the Materials and Methods. The results are presented as the means percent granule exocytosis from three independent experiments. Error bars represent the S.D. The sublines are not significantly different from each other.


1.20
1.00
0.80
0.60
0.40
0.20
0.00
O.D. (405nm)
NK3.3
NK3.3R1
NK3.3R2
NK3.3R3
NK3.3 Sublines

the Ras^{Arg-12} expressing counterparts. The next step was to assess the total intracellular enzymatic activity. The mean intracellular BLT-esterase activity from three independent experiments is shown in Figure 39. The figure shows that the NK3.3 cells that express the activated form of Ras have an increased total intracellular BLT-esterase activity as compared to the parental cell line. The differences are significant as measured by a paired "t" test for the three experiments. The results shown in Figures 38 and 39 suggest that the expression of the activated form of Ras increases total intracellular granule content in NK3.3 cells but the percentage granule release is the same for each of the cell lines.

Summary of Results

In summary, the experiments described in this thesis resulted in the following conclusions concerning the involvement of $p21^{ras}$ in natural killer cell cytotoxicity:

I. Inhibition of farnesylation, by B956, inhibited NK3.3 mediated cytolysis against the NK-sensitive tumour cell K562.

II. Stable expression of the ras^{Arg-12} gene was obtained in the NK3.3 cells, via the transduction with MSCV-v-H-Ras^{Arg-12}.

III. NK3.3 cell lines which constitutively expressed the activated Ras mutant Ras^{Arg-12}, with the occasional exception of NK3.3R2, were more cytotoxic than the controls against K562 and Raji.

IV. NK3.3R1 and NK3.3R2 expressed higher amounts of v-H-Ras mRNA than NK3.3R3. In addition, NK3.3R3 expressed different species of mRNA.

V. The NK3.3 cell lines transduced with v-H-Ras^{Arg-12} had a higher proliferation index in response to IL-2.

VI. The transduction of v-H-Ras$^{Arg\text{-}12}$ in NK3.3 cells resulted in increased IL-2 production, but no increase in the production of IFNγ or TNFα.

VII. The NK3.3 cell lines which expressed the Ras$^{Arg\text{-}12}$ mutant formed fewer conjugates with K562 than did the controls, in spite of showing higher cytotoxicity.

VIII. The constitutive expression of v-H-Ras$^{Arg\text{-}12}$ in the NK3.3 cells resulted in increased expression of CD16 and CD56, but lower expression of CD25. No changes in CD49d and e was observed. However, an increased expression of CD49f in NK3.3R3 occurred.

IX. Increased intracellular BLT-esterase activity, but not the percentage of granule exocytosis, was correlated with the expression of v-H-Ras$^{Arg\text{-}12}$ in NK3.3.

These data support the hypothesis that activation of the Ras pathway is sufficient for natural killer cell cytotoxicity.

DISCUSSION

The hypothesis of this thesis was that the expression of $p21^{ras}$ increases the cytotoxic capability of human natural killer cells. To test this hypothesis, $p21^{ras}$, activation was manipulated by chemical inhibition and by transfection of an activated $p21^{ras}$ mutant into the human NK clone NK3.3. The use of an NK clone for these studies was necessitated by the need for large numbers of a homogeneous population of cells for transfection.

The NK3.3 clone was established in culture by Dr. J. Kornbluth from a primary mixed lymphocyte culture (Kornbluth *et al.*, 1982). This clone lacks T cell markers, and lyses the traditional NK target K562. Furthermore, it exhibits morphological and marker characteristics of LGL, is IL-2 dependent, and can reach high densities in culture. NK3.3 has been used by others in experiments involving signal transduction in NK cells (Umehara *et al.*, 1997). Because of these qualities the NK3.3 clone was chosen as the recipient of gene transfer in the experiments described in this thesis. The NK3.3 cell line starts as a highly cytotoxic cell line, as confirmed by others, exhibiting cytotoxicity higher than PBL-NK cells. However, its cytotoxicity declines when cultured for long periods of time (Fig. 1, 2).

The $p21^{ras}$ protein is ubiquitously expressed in all tissues, including NK cells (Galandrini *et al.*, 1996) and NK3.3 cells (Fig. 13). This protein is activated via a large array of cellular stimulations, including cross-linking of T cell receptor and the IL-2 receptor, the B cell receptor, and an increasing number of growth factors. In NK cells $p21^{ras}$ is activated by CD16 cross-linking and when IL-2 binds to its receptor (Galandrini *et al.*, 1996). Recently, it has been reported that cross linking of the killer activatory receptor (KAR), activates the MAPK which has been classically put downstream of Ras in the signal pathway mediated by this protein (Campbell *et al.*, 1998). Based on these observations it has become clear that the activation of $p21^{ras}$ may be an important event in

the NK mediated cytolysis. The experiments described in this thesis focus on the effect of the expression of an activated $p21^{ras}$ mutant on several facets of NK3.3 function, and the effect of inhibition of the protein's function.

It has been shown here, for the first time, that the inhibition of farnesyl transferase by the B956 compound inhibits NK3.3 mediated cytotoxicity (Fig. 3, Fig. 4). This compound is believed to inhibit $p21^{ras}$ anchorage to the membrane. It has been shown by others that $p21^{ras}$ is absent from membrane preparations in NIH3T3 cells treated with the compound (Nagasu *et al.*, 1995). Others, however, claim that the effect that is seen by using farnesyl transferase inhibitors is due to other proteins such as Rho, that undergo the same post-translational change as Ras (Koch *et al.*, 1992). The lack of availability of the reagent prevented the identification of the inhibitor's true target, or confirmation of the inhibition of Ras function and membrane anchorage. However, based on others' findings, it is reasonable to hypothesize that the inhibition of cytotoxicity seen in the NK3.3 cell line is due at least in part to Ras inhibition. When tested on PBL-NK cells, no inhibition could be seen, at the concentrations previously used, when compared to the DMSO controls (Fig. 4). This result is due to the marked inhibition of DMSO alone on PBL-NK cytotoxicity. The inhibition of NK cytotoxicity by DMSO was reported by others who have documented that PBL-NK cytotoxicity is inhibited by compounds defined as hydroxyl radical scavengers, such as DMSO (Suthanthiran *et al.*, 1984). The differences in susceptibility to DMSO between NK3.3 and PBL-NK may be due to the activation state of NK3.3 *versus* PBL-NK cells, although this would be difficult to rationalize in our experiments, since the PBL-NK were also grown in culture for 48 hr in the presence of IL-2 and DMSO or B956 dissolved in DMSO. The other possibility is that the differentiation stage of the NK3.3 clone, is different than the majority of PBL-NK present in adult peripheral blood. This explanation may be correct due to the fact that the clone was established *in vitro* from a healthy donor, and due to the clonotypic nature of NK3.3.

I. Expression of Constitutively-Activated Ras in NK3.3

The first two objectives of this thesis, and the major tasks, were to obtain NK3.3 derived clones that express several mutants of $p21^{ras}$ - constitutively active mutant Ras^{Arg-12}, Ras^{Leu-61} under the control of the rat metallothionein promoter, and the dominant negative mutant of Ras, Ras^{N-17}, under the control of the same inducible promoter.

In order to understand the involvement of Ras in the cytolytic function of NK cells, it was felt that specific inhibition of the function of the protein should be demonstrated. The Ras^{N-17} mutant, described first by Cai *et al.* (1990), is a dominant negative mutant of Ras. This dominant negative mutant needs to be expressed under the control of an inducible promoter, since constitutive inhibition of cellular Ras will result in the cell's death. The only vector available at the time was MMTV-N17 (Cai *et al.*, 1990), in which Ras^{N-17} is controlled by the MMTV promoter. The MMTV promoter is induced by the presence of dexamethasone, which was found to be highly inhibitory of NK3.3 function (Fig. 8), in agreement with results in the literature (Migliorati *et al.*, 1994).

Creation of a vector containing the dominant negative mutant of Ras was supposed to be a simple task, in which the DNA fragment encoding for the Ras mutant is released from the vector by digestion with the restriction enzyme *Bam*HI, and ligated into *Bam*HI sites created by digestion of the receiving vector with the same enzyme in order to release the activated Ras fragment in the vector. All clones were found to contain the insert in an inverted orientation following ligation. Under the conditions in which the ligation was performed, there was an equal chance for the DNA fragment to integrate in one orientation or the other. The fact that there was a preference for the fragment to integrate in undesirable orientation cannot easily be explained.

NK3.3 cells were electroporated with a construct containing an activated Ras mutant Ras^{Leu-61}, under the control of the rat metallothionein promoter. This is an inducible promoter positively induced by the presence of heavy metals. In the literature this promoter is induced with Cd^{++}, Zn^{++}, or a combination of both (Andersen *et al.*, 1990;

Owaki *et al.*, 1993). Treatment of NK cells with Cd^{++} was highly toxic to the cells, in accordance with findings in other systems (Xu *et al.*, 1996). The treatment of NK3.3 with Zn^{++} had no effect on cell viability. When the cells were treated with Zn^{++} prior to a cytotoxic assay, at low Zn^{++} levels a slight inhibition of the cytotoxic function was observed. At concentrations comparable with those used for the induction of the promoter, increased cytotoxicity was observed (Fig. 9). The results in the literature are contradictory. One study on mouse NK cells shows marked inhibition of NK function in the presence of 10 – 40 μg/ml Zn^{++} (Stephenson *et al.*, 1985). No concentrations of zinc higher than these were used in this study. On the other hand, others have reported that treatment of human PBL with Zn^{++} augments NK activity (Bulgarini *et al.*, 1989). These discrepancies may be due to differences in human *vs* murine NK, or to the concentrations of zinc used in the experiments.

Lymphocytes in general, and NK cells as part of the lymphoid lineage, are difficult to transfect. Despite these difficulties 400 G418 resistant clones were obtained when the cells were transfected by electroporation with a construct containing the activated mutant of Ras under the transcriptional control of the rat-metallothionein promoter, and co-transfected with a construct containing the G418 resistance gene. When screened for induction of the Ras protein by treatment of the cells with zinc, an inducible clone was not found in the first 50 clones screened. One of the problems in using the rat-metallothionein promoter is the possibility of leakage, *i.e.*, expression of the protein transduced into the cells without the addition of inducers. However, in the MT-Ras-NK3.3 clones, despite the resistance to G418, the levels of Ras expression were much lower than in the 2H1 cell line, in the presence or absence of inducers (Fig.14). Another explanation for the phenomenon could be that, due to some selective pressure the PGK-neo construct coding for the G418 resistance was transfected into the cells without the MT-Ras construct, resulting in G418 resistant clones that do not have the activated mutant of Ras integrated into the genome. An attempt to avoid this problem was made by

the construction of an expression vector containing the activated mutant of Ras under the control of the rat MT-I promoter, and the neomycin resistance under the control of the PGK promoter on the same construct. Unfortunately, this attempt was not fruitful due to difficulties in obtaining accurate restriction maps from the investigator who provided us with the MT-I-H-Ras vector.

One of the methods used by many investigators for stable transduction of genes into haematopoietic cells is the use of retroviral vectors. High efficiency of transduction is usually achieved by this method, however the stability of the gene transfer over time is not clear. Furthermore, the levels of gene expression may change with time and growth conditions. Three NK3.3-Ras expressing cell lines were obtained from three independent infections using a construct containing the $Ras^{Arg\text{-}12}$ mutant under the control of the murine stem cell virus LTR. When compared to other retroviral promoters, this promoter was found to be relatively stable when cells are grown in culture (Lu *et al.*, 1996). Expression of the $Ras^{Arg\text{-}12}$ mutant was observed in all three cell lines at the mRNA level (Fig. 15). Interestingly, the cell line NK3.3-R3 had the lowest level of expression and two species of mRNA were visible, one of 2.9 kb and the second one of 3.6 kb, corresponding to the spliced and non-spliced species of RNA driven from the LTR promoter. This result is interesting because of the way the NK3.3-R3 was obtained, *i.e.*, the IL-2 was not present at the time of infection and the cells were grown for 7 days in the absence of the cytokine. It has been reported in the literature, using stem cells, that the presence of growth factors in the media may affect the expression of genes regulated by the MSCV promoter *i.e.*, promoter activity and gene expression in these cultures were inversely correlated to the presence of growth factors and the proliferation rate of the cells (Lu *et al.*, 1996). One possibility is that in NK cells, as opposed to stem cells, the presence of IL-2 increases the promoter activity, and therefore higher transcriptional levels were observed in the cells when the cytokine is added. Another reason for the discrepancy may be the relatively more stable environment of a fully differentiated cell as opposed to an

actively differentiating cell. In the latter, the promoter may be exposed to changes in DNA transcription due to active processes of methylation, which occur to less extent in a fully differentiated cell (Taylor, 1993). The fact that the spliced form of RNA is present in NK3.3R3 and is not present in NK3.3-R1 and NK3.3-R2 may be due again to the fact that IL-2 was absent at the time of infection in the NK3.3-R3 cell line and for the following week after the infection. These cells were not entirely IL-2 independent, because the proliferation was slowed dramatically when the cytokine was withdrawn. However, the spliced form of v-Ras may have given the cells a survival advantage in the conditions in which they were grown. It has been shown that splicing of the region between exon 3 and 4 of c-H-Ras affects its expression and transforming activity (Abrams *et al.*, 1996). Interestingly, exon 4, which is subject to alternate splicing in cellular H-Ras, contains an intron which dictates its expression levels (Cohen *et al.*, 1989). This intron is present in v-H-Ras^{Arg-12}, and can be subject to alternate splicing as well giving rise to a truncated form of mRNA, as seen in NK3.3-R3 (Fig. 15). Furthermore, a splicing event caused by the presence of the LTR viral promoter has also been reported (Trusko *et al.*, 1989). In addition, alternative splicing gives rise to a truncated form of N-Ras (Doniger, 1988). Whether there are functional differences between the two isoforms it is not clear, and further investigation of the phenotype of this cell line and the RNA species may elucidate this.

The fact that there are different levels of expression of Ras is worth noting as well. It has been shown previously that the level of expression of Ras may affect the protein's function. For example, different levels of H-Ras^{Leu-61} are needed for transformation and closure of GAP junctions in 2H1 cells (Brownell *et al.*, 1997; Raptis *et al.*, 1997). In NK3.3 cells, different signals may be transduced by the protein when high levels of v-H-Ras^{Arg-12} or lower levels are expressed.

II. Characterisation of the NK3.3R1, R2 and R3 Sublines

The second part of the thesis deals with experiments designed to characterize the Ras^{Arg-12} transduced cell lines. We found that the transduction of Ras^{Arg-12} into NK3.3 cells increases the cytotoxic function of the cells (Fig.. 17, 18). To our knowledge, this is the first report of "direct" effect of Ras activation on the cytolytic function of NK cells. Previous studies have shown that cellular Ras is activated by the cross-linking of receptors known to activate NK cells and increase NK cytototoxicity. Two such receptors are CD16 and IL-2 (Galandrini *et al.*, 1996). However, the activation of Ras in these instances may be or may be not a crucial event in the signalling mediated by the two receptors as far as the cytotoxic function of the cells is concerned, since other intracellular mediators are activated by the same receptors (Stahls *et al.*, 1994; Meyer *et al.*, 1997; Cerboni *et al.*, 1998). The results presented in Fig. 16 and 17, show that the signals mediated by $p21^{ras}$ activation are enough to activate the cytolytic machinery above the level seen in the cells in the presence of IL-2 alone. Furthermore, Figures 18 and 19 show that after long term-culture the Ras^{Arg-12} transduced cells maintain their cytotoxicity much longer than the controls. This result suggests that a flaw in the Ras pathway may be at least in part responsible for the fast decline in NK3.3 cytotoxicity in vitro, similar to the situation seen in T cell anergy, which is mediated by a blockage in the Ras mediated pathway (Fields *et al.*, 1996). However, Ras is activated in the non-cytotoxic NK3.3 when the cells were stimulated with IL-2 (data not shown). It is possible that, although Ras can still be activated by IL-2, upon interaction with the target other moieties which may activate Ras fail to do so, and therefore the activation of Ras by the introduction of Ras^{Arg-12} corrects for the dysfunctional receptor(s). Such activatory receptors which activate NK cells, and at the same time activate the $p21^{ras}$ signalling pathway, are CD44, KAR, and CD28 (Azuma *et al.*, 1992; Tan *et al.*, 1993; Galandrini *et al.*, 1994; Sconocchia *et al.*, 1994; Teng *et al.*, 1996; Campbell *et al.*, 1998).

NK3.3-R3 proved to be the most interesting cell line of the three. It exhibits the highest cytotoxic function, and at the same time has a lower level of expression of v-H-Ras^{Arg-12} at the mRNA level, as well as the spliced form of mRNA, which is absent in the other cell lines (Fig. 17, 15). This result indicates that lower levels of constitutive Ras activation perhaps correlates with increased cytotoxic function in NK3.3 cells. On the other hand the presence of a second mRNA band indicates the presence of a second isoform of v-H-Ras^{Arg-12} in the cell line. Recently, developmental regulation of K-Ras isoforms, achieved by alternate splicing has been reported (Pells *et al.*, 1997). Therefore, it is possible that the different isoforms of v-H-Ras^{Arg-12} activate different downstream effectors, and subsequently, different genes.

When stimulated with IL-2 concentrations of 500-1000 U/ml NK cells become "LAK" cells (Trinchieri, 1989). As a consequence, the target repertoire of the cells increases, leading to lysis of cell lines and tumours not lysed by NK cells. Crosslinking of the IL-2 receptor activates the signaling pathways mediated by JAK-STAT and the Ras-Raf-MAPK pathway (Graves *et al.*, 1992; Kawahara *et al.*, 1995; Winter and Long, 1997). Therefore it was hypothesized that by activating Ras the phenotype of the NK3.3 cells would be closer to that of a LAK cell. Expression of the activated mutant of Ras in the NK3.3 clone resulted in an increased cytolytic ability of NK3.3-R1 and NK3.3-R3 against the NK-resistant but LAK-sensitive cell line Raji (Fig. 21). However, when compared to LAK cells derived from peripheral blood, it was observed that the NK3.3 transduced with Ras^{Arg-12} are somewhat less cytotoxic than PBL-LAK cells. The activation of Ras alone is not enough to confer the cells with a LAK phenotype. The picture is further complicated by the fact that NK3.3 cells are IL-2 dependent, which makes them different than a freshly isolated PBL-NK cell. Despite these factors, it seems that Ras activation was able to push NK3.3 cells closer to the LAK phenotype as compared to the NK3.3 controls. Interestingly, the NK3.3 subline NK3.3-R2 does not show an increased cytolytic function against Raji. Furthermore, although higher than the

control, NK3.3-R2 is also the least cytotoxic of the Ras^{Arg-12} cell lines, when the cytotoxic function was measured against K562 (Fig. 17,18). It is difficult to interpret this result, in light of the expression of v-H-Ras^{Arg-12}, which is not different than in the NK3.3-R1 cell line. This cell line was obtained from the same batch of NK3.3, and the same packaging line was used for the infection. Because of the random integration of the retroviral vector, it is possible that the function of genes important for cytolytic function was disrupted. This would be a more feasible explanation if the NK3.3-R2 were a clone, as opposed to being a population of cells infected at the same time with the retrovirus. Whatever the mechanism may be, analysis of activation status of downstream effectors such as MEK, MAPK, or PI-3 kinase may provide some clues to the signaling events that lead to the differences in the cytotoxic function of the cell line.

Ras is in the signalling pathway mediated by a large number of growth factors for the activation of transcription factors that mediate cell proliferation (Baker *et al.*, 1994; Okuda *et al.*, 1994). Furthermore, C3H10T1/2 fibroblasts can proliferate in serum free media when transformed by v-H-Ras, in an N-Ras dependent manner, and activated H-Ras activates c-Fos serum response element in endothelial cells (Herman and Simonson, 1995; Hamilton and Wolfman, 1998). Growth factor independence following transfection of cells with an activated mutant of Ras has also been reported (Kim H.A., 1998,(Peehl *et al.*, 1997). In numerous systems Ras is able to replace the signals provided by growth factor stimulation for cellular proliferation (Maher *et al.*, 1994; Okuda *et al.*, 1994; Choudhury *et al.*, 1997; Gomez *et al.*, 1997; DeSilva *et al.*, 1998). In our system, the NK3.3-R1, R2, and R3 maintain the responsiveness to IL-2 (Fig. 24, 27). An interesting phenomenon is that the NK3.3 control cell line is able to proliferate in response to serum alone, and the addition of IL-2 fails to stimulate the cell line any further (Fig. 23). This phenomenon could have been explained by the fact that after long-term stimulation with IL-2 as the growth factor, the cells began to secrete cytokines in an autocrine fashion. As known, upon IL-2 stimulation NK cells secrete a number of cytokines such as IL-2, IL-5,

and IL-12, which are able to maintain NK proliferation *in vitro* (Trinchieri, 1989; Diaz *et al.*, 1993; Jewett and Bonavida, 1994). However, although slightly higher than the freshly thawed NK3.3 cells, there was not significantly higher IL-2 production by these cells (Fig. 29), leading to the speculation that other cytokines may be secreted. The fact that the transduction of an activated Ras mutant into the NK3.3 cells did not result in interleukin independence in the cells suggests that the signals mediated by the STAT1, STAT 3 and 5, which are also activated by the ligation of IL-2 to its receptor, may be important for the cell proliferation. The dependence of cellular proliferation upon STATs activation has been shown by others in myeloid cells or T lymphocytes, in response to IL-3 or IL-2 respectively (Takemoto *et al.*, 1997). Interestingly there seems to be a correlation between the levels of v-Ras expression and the proliferation of the cells in the presence of serum, but not in response to IL-2. Higher levels of Ras expression increase the proliferation rates of the cells in NK3.3-R1 and R2 (Fig. 24). However, the cytotoxic function of the cells is increased only slightly in the two cell lines (Fig. 22). When lower levels of v-Ras are expressed the cells do not proliferate more than the controls in the absence of IL-2, but they respond strongly to the cytokine stimulation. These results suggest that in the presence of certain levels of activated Ras the cells are able to proliferate to some extent independently of IL-2. However, the IL-2 signal is still needed for the optimal proliferation of the cells, suggesting a synergistic effect and perhaps cooperation between the STAT mediated and the Ras mediated pathways. The picture is complicated by the continuous change in culture of the control cell line, and the fact that the control itself seems to become IL-2 independent after long-term in culture. Despite the change in the phenotype, it is clear that transduction of the cells with the activated form of Ras maintains the responsiveness of the cells to IL-2.

The lack of responsiveness to IL-2 in the NK3.3 control after long term in culture is accompanied by a change in the expression of the high affinity (α) p55 chain of the IL-2 receptor (CD25). In NK cells the α chain is induced upon activation of the cells with

IL-2 or by crosslinking of CD16 (Anegon *et al.*, 1988; Trinchieri, 1989). Interestingly, in the control NK3.3 the expression of this protein is increased, as shown by flow cytometry (Fig. 37). A change in the density of the protein, and in the percent of positive cells in the population is observed (Fig. 37. Table 1). This change in the expression of the receptor correlates with the phenotypic change in cytotoxic function of the cells (Fig. 1, Fig. 17). The NK3.3 cell lines expressing the activated form of Ras do not all exhibit this change to the same extent. An increased expression and percent positive cells is observed in these cells as well, with NK3.3R1 and R2 being the most similar to the control. However, the NK3.3R3 cell line which is the most cytotoxic, and the most responsive to IL-2 both in proliferation and cytotoxic function, exhibits two distinct populations, a high density and a low or negative population (Fig. 37). CD25 downregulation has been shown on the surface of activated tumour infiltrating lymphocytes leading to decreased proliferation in response to IL-2 (Sheu *et al.*, 1997). In NK3.3 upregulation of the receptor expression is linked to the cells being refractory to IL-2 stimulation as shown by proliferation assays and cytotoxicity (Fig. 23, 19) Release of soluble CD25 has been also reported in NK cells activated with IL-15 (Treiber-Held *et al.*, 1996). It is possible that release of the receptor to the culture medium also occurs with NK3.3 cells grown long-term in culture, interfering with the binding of the cytokine to the cell bound receptor. Sorting experiments by flow cytometry could determine if there is a difference in the cytotoxic function between the high expressors of CD25 and the low expressors, and correlate this phenotype with the expression of v-Ras, which may differ in the infected population. It is not clear how the expresion of an activated Ras mutant maintains the responsiveness of the cells to IL-2 stimulation, and at the same time maintains low expression of CD25 on a certain percent of the cells in the population, similar to the highly cytotoxic freshly thawed NK3.3, and similar to non-activated PBL-NK. It is also not clear whether the other subunits which are required for the high affinity functional receptor are differentially expressed on the NK3.3 cell lines. Flow cytometry experiments with

antibodies specific for the three subunits could elucidate this. Furthermore, although present, the IL-2R may fail to activate downstream kinases such as JAK1 and 3, which in turn activate the STATs proteins. It has been shown that the MAP kinase pathway is involved in STAT regulation, since ERK2 can phosphorylate STAT3 in vitro (Chung *et al.*, 1997). Interestingly it has been reported that IL-2 is not a potent activator of ERK2 in T cells despite its ability to activate Ras (DeSilva *et al.*, 1998). Ras activation may compensate for the inability of the IL-2 receptor to activate these downstream effectors, because of the constitutive activation of the protein which may increase the activated ERK2 concentration in the cells, and by that its interactive potential with downstream effectors.

IFNγ cytokine does not use the Ras-MAPK pathway for the activation of gene transcription, but transduces its effect by utilizing the STAT proteins (Argov and Klein, 1983). These proteins translocate directly from the cytoplasm to the nucleus following the binding of IFN to the receptor, although recent reports linked the effect of IFNα to the Ras pathway as well (Berger *et al.*, 1997). This cytokine has been shown by others to increase NK cytotoxicity in PBL-NK (Argov and Klein, 1983), but had no effect, in our experiments, on NK3.3 cytotoxicity in the control or Ras transduced cell lines (Fig. 28). Differences in the response of NK3.3 versus PBL-NK to cytokines and the signal transduction pathways mediated have been reported by others, who have shown that in primary NK cells IL-2 and IFN stimulation induced phosphorylation of STAT1, STAT3 and STAT5 while in NK3.3 a greater activation of STAT5 was observed (Yu *et al.*, 1996). Since different signaling pathways have been observed in NK3.3 and PBL-NK in response to IFN, it is expected that the cellular response and the genes stimulated in the two cell populations will be somewhat different. When the cells are starved of IL-2, the cytolytic function is diminished but not the viability of the cells, and the activation of Ras on its own is unable to confer cytotoxic function on the cells (Fig. 28). This result suggests that, although Ras is activated by IL-2 in NK cells, the activation of Ras at the

levels obtained by transduction with the retroviral vector is not sufficient to replace the IL-2 signal for cytolysis. NK3.3 and PBL-NK are often stimulated with concentrations of IL-2 higher than 100 U/ml in order to obtain optimal cytotoxicity *in vitro* (Goebel *et al.*, 1996). Stimulation of the cells with high IL-2 concentrations (500 U/ml), following starvation from the cytokine restores their cytotoxic function (Fig. 28). Once more, there are differences in the response of the control NK3.3 cell line and the Ras transduced cells to the cytokine. The Ras transduced cell lines, NK3.3R1, R2 and R3 are more cytotoxic than the controls when stimulated with IL-2, with the NK3.3R3 cell line maintaining the highest cytolytic function.

These results suggest that the different signaling pathways mediated by cross-linking of the IL-2 receptor, perhaps via STATs as well as via Ras are needed for the cytotoxic function of the cells. More than that, it seems that constitutive activation of Ras is synergistic with the IL-2 stimulation, since the cytotoxic function in response to IL-2 is increased in the Ras expressing cell lines.

The cytokine production of the cell lines was also investigated in this study. It was observed that the NK3.3 cell lines expressing an activated form of Ras, NK3.3R1, R2 and R3 secrete substantially larger amounts of IL-2 as compared to the control (Fig. 29). This finding is consistent with findings in T cells showing that a block in the Ras pathway abolishes IL-2 production in T cells (Fields *et al.*, 1996). Furthermore, the Raf/MAPKK/ERK-2 pathway seems to be essential for IL-2 gene transcription in T cells activated by jacalin (Lafont *et al.*, 1998). Consistent with its cytotoxic phenotype *i.e.*, being the most cytotoxic against K562 and Raji, NK3.3R3 secreted more IL-2 than NK3.3R1 and R2. Interestingly, the NK3.3 cell line, which has been maintained in culture for a long period of time, also secretes low concentrations of IL-2. This finding may explain the refractoriness of the cell line to exogenous IL-2 stimulation, because of the presence of autocrine IL-2. However, since the Ras expressing cell lines secrete even more IL-2, it is not clear why the phenomenon is not occurring in these cell lines as well.

When activated with IL-2, NK cells secrete IFN and TNF (Jewett *et al.*, 1996) which has often been correlated with the cytotoxic ability of this cell type. Further investigation of the cytokines produced as a consequence of Ras activation revealed that after 24h in culture in the absence of IL-2 the Ras transfected cell lines secrete lower amounts of TNF and IFN as compared to the controls (Fig. 30, 31). This result suggests that the activation of the TNF and IFN transcription in NK cells is Ras independent, and may be inhibited by Ras activation in a 24h assay. It is worth noting that the transcription of these cytokines have been previously linked to the proliferation status of the cells rather than to the cytotoxic function (Jewett *et al.*, 1996), supporting the phenotype of the Ras transfectants that are more cytotoxic than the controls, and secrete lower amounts of IFN and TNF. When supernatants were collected following incubation of the cells for 72h, higher concentrations of IFN were found in the Ras-expresing cell lines cultured in the absence of IL-2, 400 - 600 pg/ml as compared to 50 -200 pg/ml (Fig. 31). In this experiment, after 72h, no differences were found between the controls and the Ras transduced cell lines, suggesting different kinetics in the cytokine production in the Ras expressing cell lines and the control. Stimulation of the cells with exogenous IL-2 increased the production of IFN approximately two fold in all cell lines consistent with previous reports (Ye *et al.*, 1995). No differences in the cytokine production were observed between the cell lines expressing an activated Ras mutant and the control. The TNF production in the Ras transduced cell lines remained low in the NK3.3 controls and the Ras-transduced cell lines, after 72h incubation in IL-2 free medium (Fig. 32). Stimulation of the cells with IL-2 resulted in a five to ten fold increase in the TNF production in all cell lines (Fig. 33). Unlike the cytotoxic function, which cannot be restored in the control cell lines by IL-2 stimulation, the production of cytokines in the cells does not differ from that of the Ras-transduced cell lines.

Taking all these results into account, it seems that the Ras pathway is not involved in the production of TNF and IFN in NK3.3 cells. It has been recently shown that the JAK-

STAT pathway is involved in the transcription of the TNF gene in T cells and that the mouse TNF-β gene contains a STAT5 binding site (Lu *et al.*, 1998). Interestingly the NK3.3R3 cell line shows low IFN and TNF production in the experiment shown in Figures 31 and 32. Further experiments would confirm if there is a real difference between this cell line and its counterparts, and the relationship to the cytotoxic phenotype of the cell line which has been reported by others to inversely correlate with the cytotoxic function (Jewett *et al.*, 1996).

The cytolytic function of NK cells is believed to be related to the ability of the cells to recognize and bind the target cells (Trinchieri, 1989). Therefore, it has been deduced that the larger the proportion of cells in conjugates at a certain point in time, the higher the cytotoxicity of the population. The data presented in Fig. 33 show that the percentage of NK3.3 cells that form conjugates with the K562 targets differs in the NK3.3 cell lines. The NK3.3R1, R2 and R3 form fewer conjugates than the control cell line. Furthermore, the NK3.3R3 cell line forms fewer conjugates than the other Ras^{Arg-12} expressing NK3.3 cell lines (Fig. 34, 35). In addition, the percentage of NK3.3 cells which form conjugates with K562 was found to be inversely correlated with the cytotoxic ability of the cell line (Fig. 36). This result is unexpected, because the cytotoxic function of NK cells is thought to be dependent on the recognition and binding of the effector cells to the target (Lanier and Phillips, 1992). It has been shown in PBL-NK that the cells undergo a period of anergy following the binding to the target cell, after which it is believed that the cell receives an inhibitory signal (Jewett and Bonavida, 1995). However, it is possible that the Ras transduced cell lines escape the negative signal transduced to the effector cell by binding to sensitive targets, therefore being able to recycle and kill more targets in a certain period of time. If this is correct, even if at a certain point in time fewer conjugates are formed the ability of the cells to readily recycle and move to kill another target may account for the high cytotoxicity. Another explanation to the phenomenon relies in the nature of the conjugates formed. The fact that an effector is

bound to the target does not necessarily mean that there will be lysis of the target cell, if a lytic conjugate is not being formed (Lebow and Bonavida, 1990). Therefore, it is possible that, although in the control and the less cytotoxic Ras expressing cell lines there are more binders, fewer of the cells that are capable of binding to the targets are actually capable of lysis. This phenomenon may be attributable to the differential expression of adhesion molecules by the different cell lines, independent of cytotoxic function.

Analysis of activation markers and adhesion molecules on the NK3.3 cell lines show upregulation of CD56 and to some extent CD16, which correlates with the cytotoxic function of the cell lines (Table 1). The Ras^{Arg-12} expressing cell lines continue to express CD56 and CD16, which are lost in the control NK3.3 cell line which is maintained long term in culture in the presence of IL-2. The expression of these markers on natural killer cells has been previously correlated with a mature, cytotoxic phenotype (Trinchieri, 1989). The expression of activated Ras maintains the expression of these markers on the surface of the cells, and at the same time maintains the cytotoxic function. Interestingly not all CD56 positive cells express CD16 (data not shown). Others have shown that the $CD56^{+}CD16^{-}$ phenotype expressed by LGL cells isolated from bone marrow represent a less cytotoxic phenotype, with a weak response to IL-2 (Carson *et al.*, 1997). It would be interesting to separate the populations by flow cytometry in order to identify the phenotype of the Ras expressing CD56-positive, CD16-negative. NK3.3R3 exhibits the highest percentage of CD56-positive CD16-positive cells, again in agreement with its highly cytotoxic phenotype. CD25 which is an activation marker, is expressed in NK cells following stimulation with IL-2 or CD16 (Anegon *et al.*, 1988; Trinchieri, 1989). However, in the NK3.3 transduced cell lines 30 - 40% of the population do not express the high affinity chain of the IL-2 receptor (CD25). Interestingly, the control cell line grown for long period of time in IL-2 shows a shift in the expression of the antigen, *i.e.*, all cells become highly positive for the expression of the high affinity receptor (Fig. 37). The highly cytotoxic, freshly thawed NK3.3 control exhibits two distinct

populations, one highly positive and a negative population (Fig. 37). Since the expression of CD25 is a marker of NK activation, it was expected that by growing the cells in IL-2 a stimulation of IL-2Rα gene would occur. Consistent with this concept, the continuous activation of the cells with IL-2 increases the expression of the α-chain of the IL-2R. Interestingly, the expression of Ras^{Arg-12} in NK3.3 inhibits the complete shift to 100% CD25 positive expression. The activation of transcription of the IL-2R-α chain is regulated by interaction of STAT5 dimers with binding sites with the responsive enhancer in the cytokine responsive enhancer (Meyer *et al.*, 1997). Interaction between the Ras pathway and the signaling pathways mediated by the JAK-STAT proteins has been previously reported (Chung *et al.*, 1997). It is possible that the constitutive activation of Ras regulates the expression of CD25 via interaction of ERK-2 with the STAT proteins, preventing them from interacting with the DNA sequences. Another possible explanation may be that by constitutively activating the Ras pathway and its downstream effectors interference at the DNA binding level, and competition between activated ERK complexes and STAT complexes may occur, preventing the transcription of CD25 in some of the cells. It is not clear how the phenotype of low CD25 expression correlates with the cytotoxic phenotype, but it seems that high expression of the alpha-chain correlates with lower cytotoxic function and refractoriness of the NK3.3 cell line to IL-2. There are differences in the CD25 percent positive cells between the NK3.3 cell lines transduced with the activated Ras mutant as well. Thirty to forty percent of the cells in the population of NK3.3R3 are CD25 negative as opposed to only about fifteen to twenty percent of the NK3.3R1 and R2 and compared to 0% in the NK3.3 control. Therefore, it seems that the cytotoxic phenotype correlates with the lack of expression of CD25 on some of the cells in the population.

CD49 are integrin α chains that are expressed as heterodimers noncovalently associated with CD29, called also VLA proteins. CD49/CD29 mediates binding of the cells to extracellular matrix components, and cell-cell interactions. CD49d mediates

binding to Peyers patch high endothelial venules, and to laminin. CD49e and f bind fibronectin (Elices *et al.*, 1990; Hemler *et al.*, 1990). No differences in the expression of CD49d, e, and f were observed, indicating that these molecules are not affected by Ras activation, and are not related to the cytotoxic phenotype of the cells. However, in adhesion assays a decreased binding potential of the NK3.3R1, 2 and 3 to fibronectin was observed, indicating that the activation status of the integrins may be different in the Ras-transduced cell lines (data not shown).

CD11c/CD18 is a β2 integrin expressed by natural killer cells (Bellón *et al.*, 1994). The function of this molecule on NK cells is not clear. In B cells, CD11c has been implicated in proliferation and activation of the cells, and in NK cells it is expressed in conjuction with CD16, and following activation with PMA (Nagler *et al.*, 1989; Laffon *et al.*, 1991; Postigo and Sánchez-Madrid, 1993). One of the signaling pathways activated by PMA is the signal transduction pathway mediated by Ras (Williams *et al.*, 1995). The percentage of CD11c expressing cells in the population is increased in the Ras expressing cell lines from 7.8% in the control to 11.7% in NK3.3R1 and R2 and to 39% of the cells in NK3.3R3. From these results it can be concluded that the activation of Ras increases the expression of CD11c, consistent with an activated NK phenotype.

The results in Fig. 38 show that despite the increased cytotoxic ability of the NK3.3 cell lines expressing an activated mutant of Ras, no increase in percent granule exocytosis was observed. However, when the total intracellular BLT-esterase activity was measured an increased activity of approximately 20% was demonstrated (Fig. 39). Therefore, it can be concluded that Ras activation increases the intracellular amount of granzyme A, granzyme B or both as measured by the BLT-esterase activity in NK3.3 cells. Others have showed that inhibition of MAPK by the inhibitor PD98059 inhibitor, inhibits granule exocytosis by NK cells stimulated by CD16 crosslinking (Milella *et al.*, 1997). However, because of the short treatment, two hours, the inhibition seems to occur at the exocytosis step and not at the transcriptional activation of the granzymes. These

results suggest that the activation of Ras may be involved in the transcription of granzymes, which activity is upregulated by the transduction of the cells with an activated Ras mutant. However, the differences in the BLT-esterase activity cannot account for the marked difference in the cytotoxic function between the NK3.3 cell line and the Ras transduced variants. Further study of the cytolytic molecules, such as the transcription of granzyme A, granzyme B and perforin at the mRNA level may lead to exciting findings into the mechanism of the Ras induced cytotoxicity.

Conclusions and Future Work

The experiments presented in this thesis were designed to investigate the role of the $p21^{ras}$ protein in the function of NK cells, using as the model system the well described NK3.3 clone.

The farnesyl transferase inhibitor (B956) inhibited the NK3.3 mediated cytolysis by 50% at the concentrations used, a result compatible with an involvement of $p21^{ras}$ in NK cytotoxicity. Implications for the phenomenon in chemotherapeutic usage of such inhibitors, and their possible effect on the immune system should be taken into consideration.

Stable cell lines expressing an activated mutant of Ras, $Ras^{Arg\text{-}12}$, have been obtained using a retroviral expression vector, which made this project possible. The presence of IL-2 at the time of infection of the cells with the retroviral vector affects the level of the expression of the gene under the regulation of the LTR of the murine stem cell leukemia virus, and possibly the splicing.

The expression of an activated Ras mutant increased NK3.3 mediated cytotoxic function, as measured against the NK-sensitive target K562, and the NK-resistant but LAK sensitive cell line Raji. The increased cytotoxic activity of the cells expressing the activated Ras mutant against Raji suggests that Ras is involved in the IL-2 stimulation of NK cells to become LAK cells.

The phenotype of NK3.3R3, one of the Ras^{Arg-12}-transduced cell lines, is extremely interesting. It is the most cytotoxic, but at the same time it expresses the lowest v-Ras amounts as seen by Northern Blot. Furthermore, a spliced form of mRNA is present only in this cell line, indicating possible different intracellular interactions with downstream effectors. Further investigation in the signaling pathways in these cell lines, such as the activation of ERK1/ERK2, PI-3 kinase, the Rho proteins and RalGDS will add to our understanding of this phenotype, as well as the understanding of the signal transduction pathways which mediate the cytotoxic function of natural killer cells.

The differences in the response to IL-2 of the NK3.3 cell lines transduced with the activated form of Ras is also interesting and worth further investigation. It is not clear how Ras interacts, if at all, with the STAT proteins which are activated by the IL-2 receptor when it is bound to its ligand. Furthermore, the involvement of Ras in the regulation of the expression of CD25 is a novel observation. Future studies will add to the understanding of this phenomenon, and the molecular interactions involved.

The expression of activated Ras induces at least a two fold increase in the IL-2 production in NK3.3 cells, in conformity with previous studies in T lymphocytes (Baldari *et al.*, 1992). NK3.3R3, which expresses less of the v-Ras, secretes the highest amount of the cytokine. It is possible that the high cytotoxic function of this cell line is due to the autocrine stimulation. Inhibition experiments with an anti-IL-2 antibody could answer this question. TNF and IFN production is not affected by the presence of activated Ras, suggesting that the Ras pathway, at least at the levels of expression in these cell lines, is not involved in the trascriptional activation of these genes.

The NK3.3R1, R2 and R3 form fewer conjugates with K562 than the NK3.3 control cell line. This finding seems paradoxical, but it can be explained in few ways. First, not all conjugates are lytic conjugates, and some NK cells which are called "binders" are not necessarily killers (Lebow and Bonavida, 1990). Second, the ability of the cells to recycle from target to target may be different or defective in the non-cytotoxic

control, and at any point of time less cells are bound, but the lysis of the targets is actually more efficient in the cells that form fewer conjugates. Further studies of the kinetics of the conjugate formation of the cell lines and the lytic conjugates formed by each of the cell lines will help us to understand this paradoxical phenomenon.

The expression of activated Ras in NK3.3 increases the expression of some of the markers linked to an activated NK or mature functional phenotype. CD16, CD56 have been linked to a functional mature NK phenotype and the percent positive is increased in the Ras expressing cell lines. No difference in CD49c, d, and e was observed and a downregulation of the adhesion potential of the cells was observed in the v-Ras expressing cells, suggesting a role for Ras in the regulation of the activation, but not in the expression, of these integrins,.

The expression of Ras increases the BLT-esterase activity in NK3.3, but not the percent exocytosis. Others have showed that the activation of ERK2 is necessary for granule exocytosis following CD16 crosslinking in PBL-NK cells (Milella *et al.*, 1997). However the activation of Ras fails to increase this process, suggesting that the activation of this protein may be directly linked to the transcription of the granzymes A and/or B therefore imcreasing the total intracellular content. Further investigation of the differential expression of the granules content, could elucidate the exact role of Ras in transcriptional activation of the lytic molecules.

The experiments described in this thesis, and primarily the establishment and characterization of the NK3.3 cell lines expressing $Ras^{Arg\text{-}12}$ are the ground work for the investigation of the $p21^{ras}$ protein in the function of natural killer cells. The results presented show that constitutive Ras activation is sufficient to maintain the cytotoxic phenotype of the cell lines under the conditions described, and opens new avenues of research in the effort to understand the signal transduction pathways which activate the cytolytic machinery of natural killer cells.

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Marital Status	Married with three children
Citizenship	Canadian

Education

1992-present	Ph.D, Department of Microbiology and Immunology, Queen's University, Thesis: The role of p21ras in Natural Killer Cell function, under the supervision of Dr. H. Pross.
1986-1988	M.Sc. Department of Microbiology and Immunology, Tel-Aviv University, Israel. Thesis: Effect of biological inducers on polyoma virus transformed 3T3 fibroblasts, under the supervision of: Dr. I.P. Witz and Dr. M. Ran.
1983-1986	B.Sc. Faculty of Life Sciences, Tel-Aviv University, Israel

Awards, Fellowships

1995-1996	Queen's Graduate Award
1994-1995	Queen's Graduate Award
1993-1994	Ontario Graduate Scholarship
1992-1993	Queen's Graduate Award
	Queen's Graduate Fellowship

Teaching Experience

Tel-Aviv University, Israel

1988-1989	Teaching assistant for the 3rd year, Immunology Labs.
	Teaching assistant for the Advanced Microbiology Labs.

Queen's University

1992-1998	Teaching assistant for Introductory Immunology of Life Sciences third

year students, and for first year Medical Immunology.

Positions Held

1988-1990	Research Assistant, Biotechnology General Ltd., Israel Department of Biological applications and preclinical testing. Performing pre-clinical testing of anti-clotting and wound healing drugs *in vivo* and *in vitro*.
1991-1992	Research Assistant, Department of Haematology, St. Michael's Hospital, Toronto. Developing of ELISA systems to asses the presence of antiidiotipic antibodies in sera from patients with thrombocytopenia purpura.

Technical Skills

Protein purification by column chromatography, radioimmunoassays, ELISA, immunoprecipitation, staining surface and intracellular antigens using flow cytometry, staining of intracellular antigens *in situ*.

Preclinical studies involving a variety of laboratory animals such as mice, rats and rabbits, in model systems for coagulation and wound healing. Removal of solid and ascites toumors from mice and removal of thymuses for establishment in tissue culture.

Gene expression in adherent cells or cells in suspension using calcium phosphate or by electroporation, and using retroviral vectors. DNA cloning , PCR, Northern blotting, Western blotting.

Kinase assays, assessment of Ras activation (GTP/GDP ratio).

Extensive tissue culture involving cells in suspension, adherent cells, of murine and human origin, and cell cloning.

Publications

Ran, M., Katz, B., Kimchi, N., Halachmi, E., Teillaud, J.L., Even, J., Berco-Flint, Y., Atlas, E., Witz, I.P. In vivo acquisition of FcγRII expression on Polyoma virus-transformed cells derived from tumors of low latency. Cancer Res 51:612-618 (1991).

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Abstracts

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Atlas, E., Juvet, S.C., and Pross, H.F. The effect of constitutive expression of activated Ras on NK function. Health Sciences Research Day, (1998) (Abst. 1).

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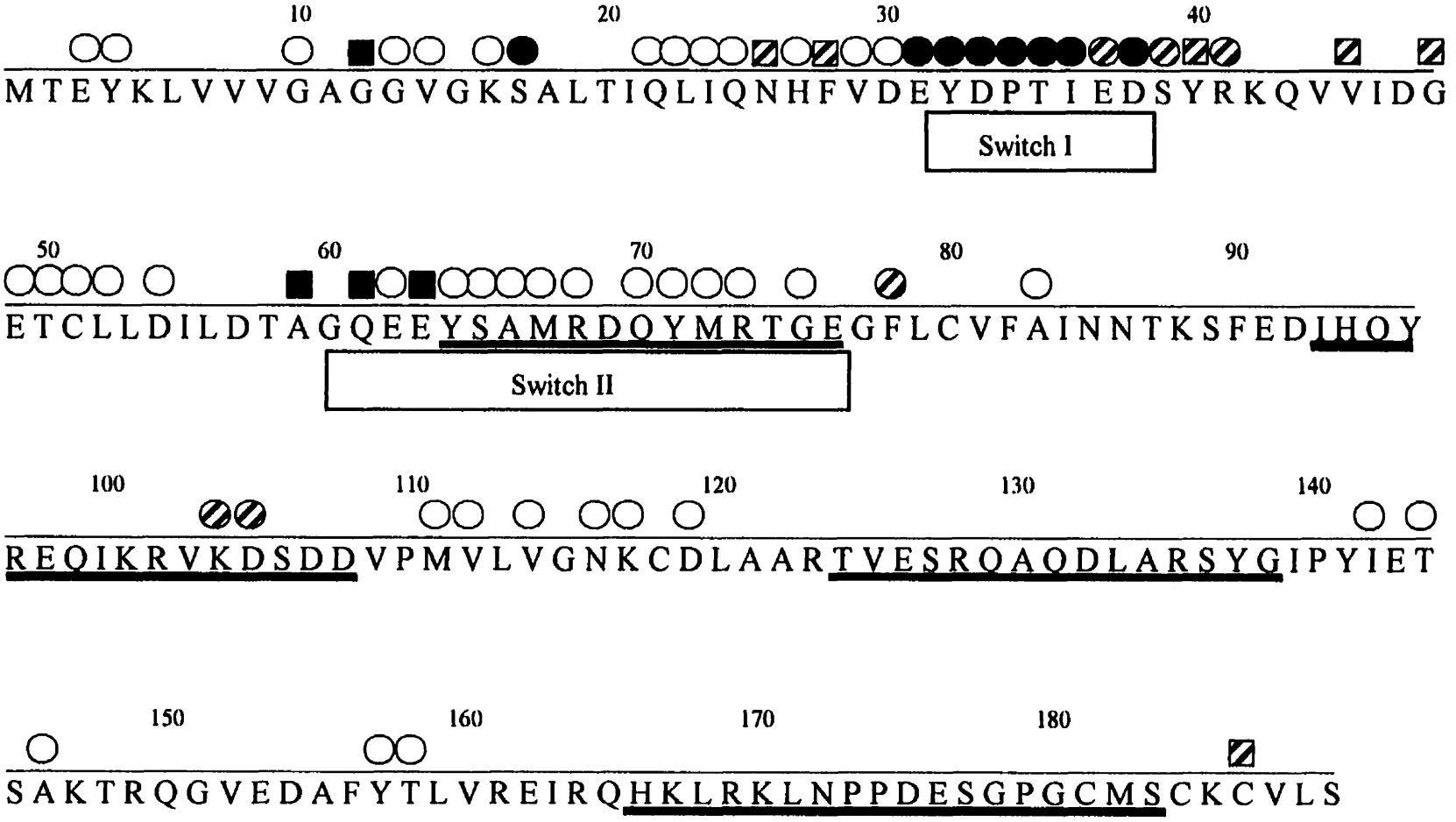
APPENDIX

$p21^{ras}$ Mutations and the Effect of $p21^{ras}$ Expression on NK3.3 Natural Killer Cell Cytotoxicity

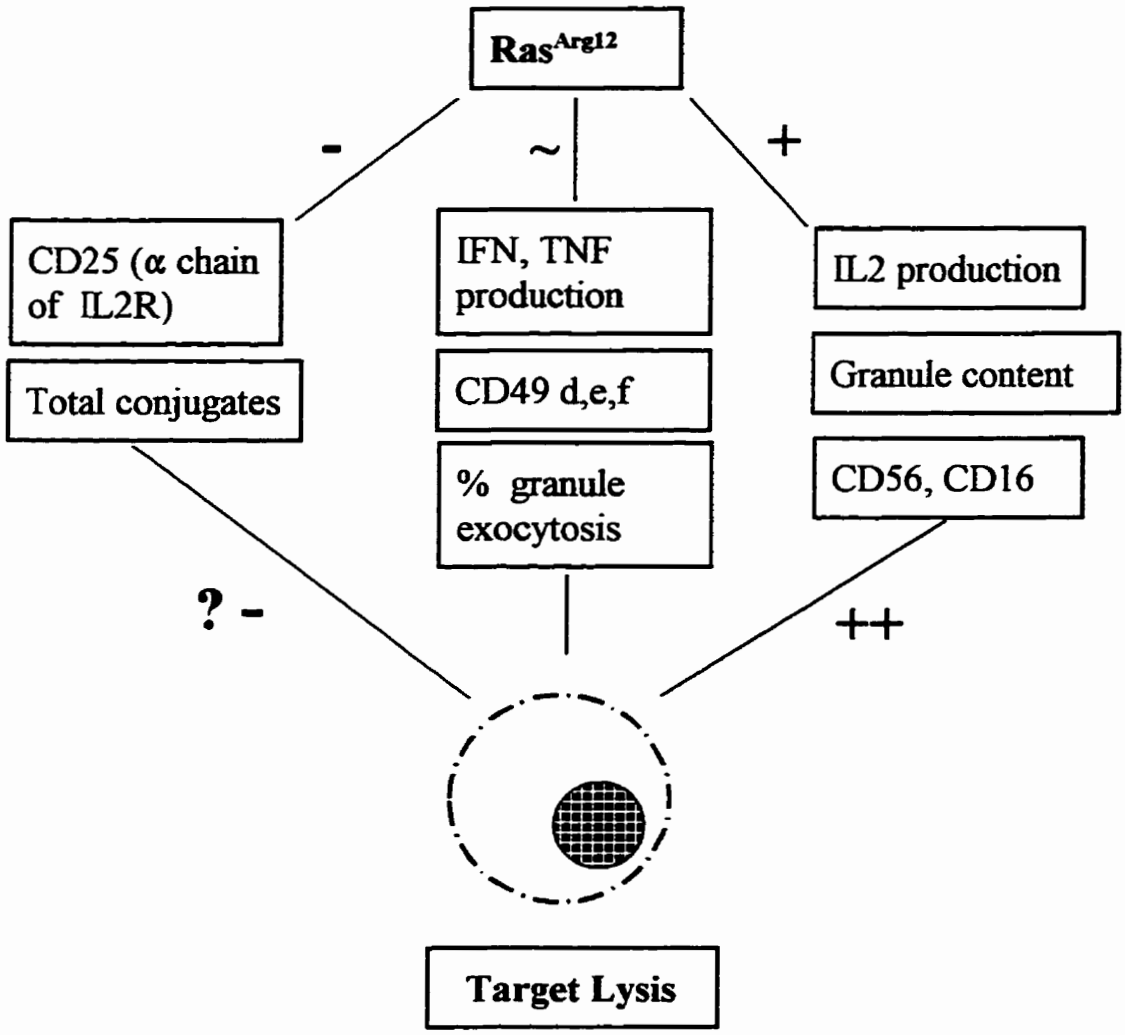
Fig. 1. Linear representation of the Ha-c-Ras protein sequence showing sites of mutagenesis and their effects. Solid black lines indicate regions that can be deleted without blocking effector function. Hatched circles, mutations without effect; Solid circles, mutations that affect both effector function and interaction with GAP and neurofibromin; Open circles, mutations with weak phenotypes; Solid squares, mutations that affect only interactions with GAP and neurofibromin; Hatched squares, mutations that affect only effector function. The switch I and II regions are indicated. Effector function is defined as positive biological effect in NIH3T3 cells, PC12 cells or S. *cerevisiae*. Interactions with GAP and neurofibromin mean either activation of $p21^{ras}$ GTPase or $p21^{ras}$ binding. Mutations at positions 12,13,16,17,35,83,116,117,119,144 and 146 can also affect intrinsic GTPase or nucleotide binding. (Adapted from **Marshall, M..S.**,The effector interactions of $p21^{ras}$.*Topics in Biological Sciences*, **18**: 250-254,1993)



Linear representation of the Ha-c-Ras Protein Sequence Showing Sites of Mutagenesis (Marshall, 1993)

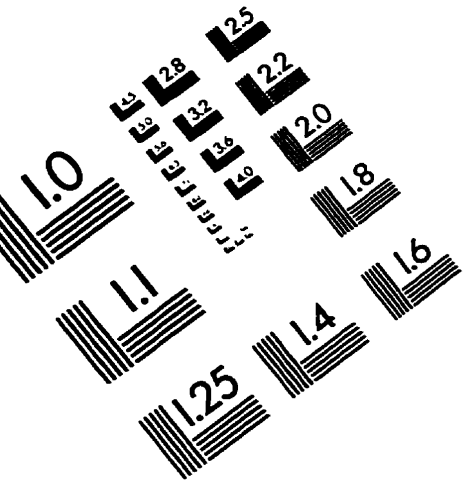
Fig. 2. Schematic diagram of the effects of the expression of $p21^{ras}$ on NK3.3 natural killer cell cytotoxicity and related functions as shown in this thesis. Positive (+), negative (-) and no effect (~) are in comparison with control NK3.3 cells maintained in culture for the same length of time as the cells expressing Ras^{Arg12}. The (?) next to the negative symbol is to indicate that it is unknown whether the lack of expression of the α chain of the IL2 receptor on some of the cells is inhibitory. The significance of a lower conjugate count and its possible effect on overall cytotoxicity is also unknown (see Discussion).

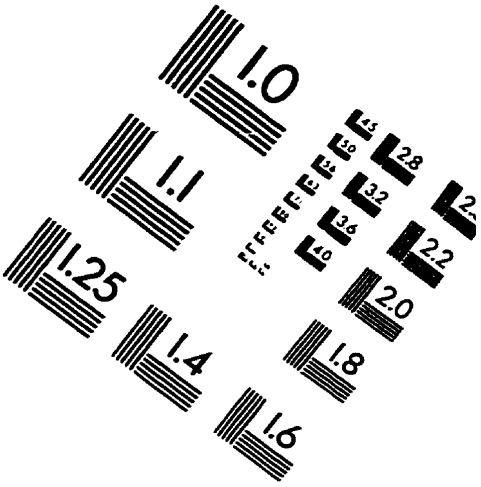
The Effect of the Expression of Ras^{Arg12} on NK3.3

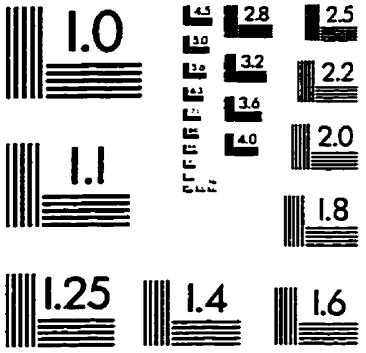


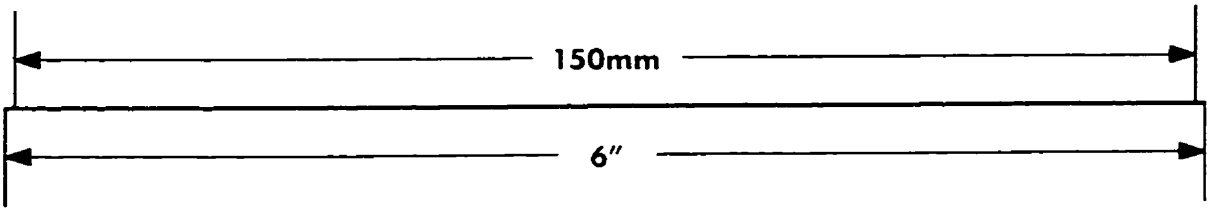
Positive (+), negative (-) and no effect (~) are in comparison with control NK3.3 cells maintained in culture for the same length of time as the cells expressing Ras^{Arg12}.

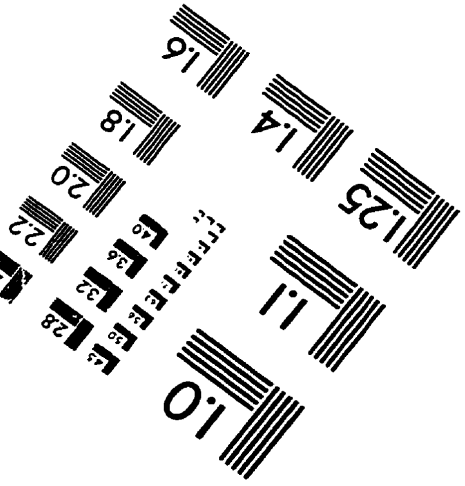
IMAGE EVALUATION
TEST TARGET (QA-3)

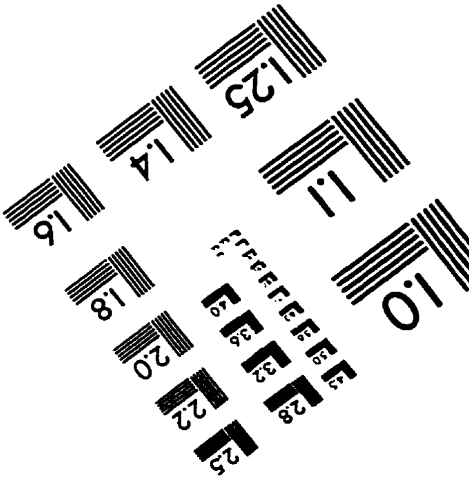












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