ANTIESTROGENS MODULATE THE PERFORIN/GRANZYME PATHWAY OF NATURAL KILLER CELL-MEDIATED CYTOLYSIS

By

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Antiestrogens Modulate the Perforin/Granzyme Pathway of Natural Killer Cell-Mediated Cytolysis

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Seyed Mohammad Mansour Haeryfar

A Practicum submitted to the Faculty of Graduate Studies of The University

of Manitoba in partial fulfillment of the requirements of the degree

of

MASTER OF SCIENCE

SEYED MOHAMMAD MANSOUR HAERYFAR©1999

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Table of Contents

Table of contents

Acknowledgements

Abbreviations

List of figures

List of tables

Abstract

Introduction

I. General Background

1. Immune Surveillance

2. Immune-derived Killer Cells

2.1. Cytotoxic T Lymphocytes (CTLs)

2.2. Natural Killer (NK) Cells

2.3. Lymphokine-activated Killer (LAK) Cells

3. Cell Death

3.1. Necrosis

3.2. Apoptosis

4. Immune Cytolysis

4.1. Fas/Fas Ligand Pathway

4.2. Perforin/Granzyme Pathway

5. Immunotherapy for Cancer

6. Antiestrogens

7. Antiestrogens and the Immune System

II. Specific Background

1. Antiestrogens and Immune Cytolysis - Review of Literature

2. K562 Cell Line

3. Antiestrogens and NK-CMC against K562

III. Rationale and Specific Goals

Materials and Methods

1. Cell Populations

1.1 Cell Lines

1.2. Separation of Human Peripheral Blood Lymphocytes (PBLs)

1.3. Preparation of Murine Splenocytes

1.4. *In Vitro* Generation of Lymphokine-activated Killer (LAK) cells

2. Drugs and Reagents

2.1. Antiestrogenic Drugs

2.2. Interleukin-2 (IL-2)

2.3. Antibodies

2.4. Inhibitors

3. ^{51}Cr Release Cytotoxicity Assay

4. Calcium Chelation by EGTA/$MgCl_2$

5. Flow Cytometry Analysis of Fas Receptor

6. Statistical Analysis

Results

I. Antiestrogens and Natural Killer Cell-mediated Destruction of K562

1. The Effect of Antiestrogenic Pretreatment Alone on NK-CMC

2. The effect of Antiestrogens on Anti-Fas Antibody-mediated Lysis of K562

3. The Effect of Antiestrogens on Fas Receptor Expression By K562

4. The Effect of Ca^{++} Chelation on Antiestrogen Modulation of NK-CMC

5. The Effect of General Caspase Inhibitor z-VAD-fmk on Antiestrogen Modulation of NK-CMC

6. The Effect of Serine Protease Inhibitor DCI on Antiestrogen Modulation of NK-CMC

II. Antiestrogens and IL-2-activated Killer Cell-mediated Destruction of K562

III. Antiestrogens and Anti-Fas-mediated Lysis of Other Tumor Targets

Discussion

I. General Discussion

II. Antiestrogens, Fas/FasL Pathway And K562 Cytolysis

III. Antiestrogens, Perforin/Granzyme Pathway and NK Cell-mediated Cytolysis of K562

IV. Antiestrogens and LAK Cell-mediated Cytolysis of K562

V. Antiestrogens and Anti-Fas-mediated Cytolysis of Other Tumor Targets

VI. General Conclusion

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Abbreviations

ADCC	: Antibody-dependent Cell-mediated Cytotoxicity
Asp	: Aspartic Acid
BRMP	: Biological Response Modifiers Program
CCP	: Cytotoxic Cell Protease
CD	: Cluster of differentiation
CLs	: Cytotoxic Lymphocytes
CMC	: Cell-mediated Cytotoxicity
CML	: Chronic Myelogenous Leukemia
cpm	: counts per minute
CSP	: Cytotoxic Serine Protease
CTLA	: Cytotoxic T Lymphocyte Antigen
CTLs	: Cytotoxic T Lymphocytes
DCI	: 3,4-Dichloroisocoumarin
DMSO	: Dimethyl Sulfoxide
DNA	: Deoxyribonucleic Acid
EBV	: Epstein-Barr Virus
EGTA	: Ethylene glycol-bis-(β-aminoethyl ether)N,N'-tetraacetic acid

ER : Estrogen Receptor

FACS : Fluorescence-Activated Cell Sorter

Fas : Fas receptor (originally Fibroblast associated)

FasL : Fas ligand

FCS : Fetal Calf Serum

FITC : Fluorescein isothiocyanate

GM-CSF : Granulocyte-Macrophage Colony-stimulating Factor

GrzA : Granzyme A

GrzB : Granzyme B

GVL : Graft-Versus-Leukemia

HBSS : Hank's Balanced Salt Solution

HF : Hannukah Factor

IFN : Interferon

IL : Interleukin

IAP : Immunosuppressive Acid Protein

LAK : Lymphokine-activated Killer (Cell)

LCM : Lymphokine Conditioned Medium

LGLs : Large Granular Lymphocytes

LPS : Lipopolysaccharide

mAb : monoclonal Antibody

$MgCl_2$: Magnesium Chloride

MHC : Major Histocompatibility Complex

MLC : Mixed Lymphocyte Culture

MLV : Moloney Leukemia Virus

NGF : Nerve Growth Factor

NK : Natural Killer (Cell)

NK-CMC : Natural Killer Cell-mediated Cytotoxicity

PBLs : Peripheral Blood Lymphocytes

PBMCs : Peripheral Blood Mononuclear Cells

PFN : Perforin

PG : Prostaglandin

RNA : Ribonucleic Acid

RPMI : Roswell Park Memorial Institute

SEM : Standard Error of the Mean

SER : Suppressive E-receptor

TALs : Tumor-associated Lymphocytes

TCR : T Cell Receptor

TGF-β : Transforming Growth Factor-beta

Th	: T helper
TILs	: Tumor-infiltrating Lymphocytes
TNF-α	: Tumor Necrosis Factor-alpha
TSP	: T cell Serine Protease
TX	: Tamoxifen
TO	: Toremifene
zAAD-cmk	: benzyloxycarbonyl-Ala-Ala-Asp-chloromethylketone
zVAD-fmk	: benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone

List of Figures

Figure		Page
1	The effect of antiestrogenic pretreatment on NK cell-mediated cytotoxicity against K562 tumor targets	39
2	The effect of antiestrogenic pretreatment on Fas receptor (CD95/Apo-1) expression by K562 human erythroleukemia cells	43
3	The effect of Ca^{++} chelation on NK cell-mediated K562 cytolysis with or without antiestrogen treatment	45
4	The effect of serine protease inhibitor DCI on NK cell-mediated K562 cytolysis with or without antiestrogen treatment	47
5	The effect of Interleukin-2 on K562 immune cytolysis	50
6	The effect of Ca^{++} chelation on LAK cell-mediated K562 cytolysis with or without antiestrogen treatment	52

List of Tables

Table		Page
1	The effect of antiestrogenic treatment on YT-INDY-mediated K562 cytolysis	41
2	Specific cytotoxicity of human NK3.3 and NKL cell lines and murine splenocytes against K562 in a 5-hour 51-Cr release assay	41
3	The effect of toremifene pretreatment on K562 cytolysis triggered by anti-Fas antibody.	42
4	The effect of general caspase inhibitor z-VAD-fmk on antiestrogen modulation of K562 immune cytolysis.	46
5	The effect of Ca^{++} chelation on K562 oncolysis induced by IL-2-stimulated PBLs or LAK cells with or without antiestrogen treatment	51
6	The effect of antiestrogenic treatment on anti-Fas-mediated cytolysis of other human tumor targets	53
7	The effect of antiestrogenic treatment on anti-Fas-mediated cytolysis of murine tumor targets	54

Abstract

Antiestrogenic drugs tamoxifen (TX) and toremifene (TO) are known to augment immune cytolysis in tumor targets induced by various killer cells. Here, we show that antiestrogens are capable of enhancing natural killer (NK) cell-mediated cytotoxicity against K562 erythroleukemia cell line and that both target and effector cells should be treated with antiestrogens in order for the maximal lysis to be achieved. K562 cells are Fas (CD95/Apo-1)-negative and treatment with antiestrogens had no influence on Fas expression. The presence of the Ca^{++} chelator EGTA/$MgCl_2$ in the cytotoxicity assay totally abrogated K562 cytolysis and its antiestrogen-mediated augmentation, suggesting the involvement of the perforin/granzyme pathway. Treatment of K562 with the general caspase inhibitor zVAD-fmk did not inhibit specific cytotoxicity, indicating the caspase-independent nature of K562 immune cytolysis. When interleukin-2 (IL-2)-activated killer cells were subjected to antiestrogen pretreatment before being used against K562, cytotoxicity was significantly inhibited. In conclusion, antiestrogens can affect the perforin/granzyme pathway of killer cell-mediated oncolysis.

Introduction

I. General Background

1. Immune Surveillance

Immunology has been called "The Science of Self", and self-nonself discrimination has been assigned as the driving force for its complex evolution (Silverstein et al, 1997). The concept of immunological surveillance against neoplastic cells was initially proposed by Erlich in 1909 and later elaborated by Burnet (Burnet, 1970 and Botti et al, 1998). Sir MacFarlane Burnet pointed out in 1970 the enormous power and complexity of the immune system, which apparently detects, invades, and destroys many cancer cells daily in the normal human body (Weinstock, 1984). The hypothesis that immunodepression favours a carcinogenetic process remains controversial in spite of numerous experimental studies. The frequent immunosuppressed condition of cancer patients at tumor relapse or recurrence of secondary tumors is a clinical sign supporting this hypothesis, and many studies have shown an impaired immune response in patients diagnosed with advanced cancer. Several mechanisms of escape from the immune surveillance have been described, including the immunoselection of tumor antigen-negative variants, the downregulation of major histocompatibility complex (MHC) class I expression, suppression phenomena and immunosuppressive cytokines and substances like transforming growth factor-

beta (TGF-β), the protein p15E, interleukin 10 (IL-10), prostaglandin E2 (PGE2), mucins, suppressive E-receptor (SER), immunosuppressive acid protein (IAP), etc. (Botti et al, 1998). However, while it is statistically true that clinical cancer occasionally occurs following development of immune deficiency, the fact remains that the most common forms of cancer (i.e. breast, lung, and colon) are not experienced by immunocompromised individuals. Inasmuch as there is scientific evidence indicating that the unprovoked, normal immune system does not recognise and destroy cancer cells that arise spontaneously, the concept of immune surveillance remains an appealing but unproven hypothesis (Green, 1993).

2. Immune-derived Killer Cells

Although a variety of immune effector cells, including macrophages, natural killer (NK) cells, lymphokine-activated killer (LAK) cells, and T lymphocytes, exhibit *in vitro* lytic activity, cytotoxic T lymphocytes (CTLs) and NK cells represent the body's primary defence against virus-infected cells or in immune surveillance against newly transformed cells (Darmon and Bleackley, 1998). These two types of effector cells which originate from the immune system differ considerably in the way they detect foreign or mutated antigens, but utilize highly analogous mechanisms for inducing target cell death (Trapani, 1998).

Lymphokine-activated killer (LAK) cells have also been a subject of intense investigations in recent years.

2.1. Cytotoxic T Lymphocytes (CTLs)

Both CD4+ Th1 and CD8+ cytotoxic T lymphocytes can deliver a cytotoxic signal upon activation and formation of conjugates with target cells (El-Khatib, *et al*, 1995). CTLs which are generated as a result of an immune response recognize and kill their targets in a major-histocompatibility complex (MHC)-restricted manner according to which only antigenic peptides displayed in the groove of an MHC molecule will be detected. CD4+ and CD8+ cytotoxic T lymphocytes recognize the antigens in the context of MHC II and MHC I molecules, respectively. In addition to the signal provided by T cell receptor (TCR) engagement, costimulatory signals are also needed for CTL activation.

2.2. Natural Killer (NK) Cells

Natural killer cells are large granular lymphocytes (LGLs) that originate in bone marrow and are able to kill certain targets including transformed and virus-infected cells with no need for prior sensitization. Natural killer cells destroy susceptible target cells in an MHC-nonrestricted fashion. In fact, NK cells are different from T cells in that they do not recognize target cells in a strictly antigen-specific manner, thereby presumably enabling a first line of defence

quickly and without antigen presentation. Moreover, they seem to recycle their lytic activity after lysing sensitive target cells (Yamauchi, et al, 1996). NK cells also express FcγRIII (CD16), which enables them to kill antibody-coated targets through antibody-dependent cell-mediated cytotoxicity (ADCC). (Vivier, *et al*, 1991). Cytokines like IL-2, IL-12 and interferons have been shown to stimulate NK cells for proliferation and/or increased cytotoxicity (Cho, *et al*, 1996 and Dao, *et al*, 1998).

2.3. Lymphokine-activated Killer (LAK) Cells

Lymphokine-activated killer cells are derived *in vitro* by culture of peripheral blood cells or tumor-infiltrating lymphocytes (TILs) from cancer patients with high doses of IL-2. LAK cells exhibit a markedly enhanced and nonspecific capacity to lyse other cells, including tumor cells. The cellular origin of LAK cells generated from peripheral blood lymphocytes (PBLs) has been a matter of controversy. CD4+ and CD8+ T cells do not develop significant LAK activity when PBLs are cultured with IL-2 or even when they are activated with a T cell stimulus such as OKT3 monoclonal antibody (mAb). After depletion of CD8+ or CD4+ from PBLs, the remaining CD4+ or CD8+ cells are able to generate non-MHC-restricted lysis of NK-resistant tumor targets upon stimulation with OKT3 and IL-2. Moreover, T cells removed from PBLs were able to generate

high LAK activity. Mixing experiments demonstrated that the CD4+ or the CD8+ cells isolated from the PBL cultures were able to inhibit the development of lytic function in the CD4-depleted and CD8-depleted cultures (Geller, *et al*, 1991). In another model using low-dose IL-2 and granulocyte-macrophage colony-stimulating factor (GM-CSF) to stimulate peripheral blood stem cells from cancer patients, LAK activity was significantly reduced by depletion of either CD4+ or CD8+ T cells and almost completely abolished after depletion of both subsets, suggesting that T cells and not NK cells were the main LAK precursors (Herrera, *et al*, 1997). By contrast, kinetic analysis used to define lytic events in peripheral blood mononuclear cells (PBMCs) activated in lymphokine conditioned medium (LCM) and IL-2 showed that T-depletion resulted in a LAK population with the highest maximum rate of lysis (LeFever, *et al*, 1991). In another study comparing growth, function and phenotype of purified NK and T cells in long-term culture to those of conventionally prepared LAK cells, it was demonstrated that the lytic activity in both conventional LAK and T- or NK cell-enriched IL-2 cultures was mediated primarily by CD56+, CD16+, CD3- NK cells (Fuchshuber, *et al*, 1991).

3. Cell Death

Cell death is the process which culminates in cessation of biological activity. In

contrast to cell quiescence or dormancy, which is also characterized by decreased rates of many biological activities, the changes that accompany death are irreversible. However, it is often difficult to define at which point a cell has passed the point of no return in the death process. The subject of cell death has recently become a focus of interest for investigators from a variety of fields. The interest expands to involve a wide range of molecular, biochemical, and morphological changes which not only pertain to the actual act of cell death, but also predispose the cell to respond to an environmental or intrinsic signal by death, regulate the initial steps leading to irreversible commitment to death, and activate the postmortem cell disposal machinery (Darzynkiewicz *et al*, 1997). Studies of dying cells have shown that there are two major mechanisms involved in cell death. These two types of death, necrosis and apoptosis, are fundamentally distinct in their morphological and biological characteristics (Leach, 1998).

3.1. Necrosis

Necrosis is a pathologically passive death occurring accidentally by extreme injury or damage to the cell (Samali *et al*, 1996). An identifying characteristic of necrosis, often referred to as accidental cell death, is the morphological change that occurs as an increase in cell volume. This cellular swelling results

from a change in the osmotic pressure gradient, which is due to loss of control of ion influx allowing the cell to take in more water. As the cell volume increases, the chromatin inside the cell takes on a more flocculated pattern with a subsequent loss of plasma and organelle membrane integrity. This eventually leads to cell rupture and the release of cellular components into the extracellular space followed by an inflammatory response. DNA degradation of necrotic cells takes place later by proteases destroying histones. The DNA molecules are then exposed to nucleases giving the characteristic smear pattern, which is seen on electrophoresis of DNA (Leach, 1998).

3.2. Apoptosis

The word apoptosis (ἀπόπτωσισ) is derived from the Greek words *apo*, meaning "apart" and *ptosis*, for "fallen", to describe the dropping off or falling off of petals from flowers, or leaves from trees. This term was first suggested by Kerr, Wyllie and Currie, a group of researchers at the University of Edinburgh, to describe distinct ultrastructural morphological changes characteristic of some dying cells (Kerr *et al*, 1972). Apoptosis is a process of programmed cell death in which a cell activates its own machinery to commit suicide. Apoptosis is essential for development and homeostasis, for defence, and for removal of aging cells. It is an active process requiring energy in the

form of ATP to maintain cellular integrity. This energy is necessary along with ribonucleic acid (RNA) and protein synthesis to fabricate the molecules needed for its successful completion. The apoptotic cell first loses contacts with its neighbours. Rapid loss of water and ions results in condensation of cytoplasm and nuclear constituents. As the nucleus begins to condense, it forms peripheral chromatin caps that line the nuclear membrane. The nucleus continues to condense until it becomes dark and pyknotic. Then complex invaginations develop in the nuclear membrane giving rise to multiple segmented nuclei. When the cell loses cytoplasmic volume, the plasma membrane becomes ruffled and blebbed. The cell then condenses into cytoplasmic proteins with its intracellular organelles remaining intact. This wrinkling zeiotic plasma membrane leads to cellular fragmentation. The fragments which are so formed are known as apoptotic bodies. When the DNA of these bodies is analysed by gel electrophoresis, cleaved bands will give a ladder appearance representing DNA fragmentation which is the hallmark of apoptosis. Each cleavage is at an interval of about 200 base pairs between the nucleosome linker regions. The purpose and the mechanism(s) involved in cleavage of DNA are not understood yet. It has been proposed that this event happens early in the process to serve as a protective measure for the nearby phagocytic cells by destroying the unwanted

potentially harmful genetic information (Leach, 1998). The importance of apoptosis is based on the fact that apoptotic cells tend to be "environmentally friendly" in a sense that they package their contents into membrane-bound vesicles, ready for ingestion by phagocytic cells, without releasing their contents into the intercellular matrix, and hence there is no inflammatory response. Apoptosis is also an altruistic type of cell death, in that damaged cells commit suicide to allow the neighbouring cells to continue to proliferate without being affected by the death of the neighbour. In addition, sacrifice of individual abnormal cells will benefit the whole organism (Samali et al, 1996).

4. Immune Cytolysis

Over the past two decades, immunologists have strenuously attempted to decipher the molecular basis of lymphocyte-mediated cytolysis. The delivery of the lethal hit by CTLs/NK cells induces apoptosis of the target cell, involving pre-lytic DNA fragmentation into oligonucleosome-length DNA fragments (Berke, 1995a). Cytotoxic immune lymphocytes are known to destroy target cells via two major mechanisms called Fas/Fas ligand (FasL) and perforin/granzyme pathways (Uslu et al, 1997). It is reported that following activation, NK cells can also acquire the ability to secrete tumor necrosis factor (TNF-α) and consequently kill TNF-susceptible targets (Vujanovic et al, 1996).

4.1. Fas/Fas Ligand Pathway

Fas (CD95/Apo-1) is a type I transmembrane protein belonging to the TNF/ nerve growth factor (NGF) receptor family (Nagata and Suda, 1995). Fas (fibroblast associated) appears to be more than a simple mediator of apoptosis. On fibroblasts, Fas ligation can lead to either proliferation or apoptosis depending on the relative number of expressed Fas molecules (Freiberg et al, 1997).In the cytoplasmic region of human Fas, there is a 68 amino acid residue-long domain called the Fas associated death domain (FADD) that, upon Fas ligation by either FasL or anti-Fas antibody, transduces the intracellular signals required for programmed cell death (Varfolomeev et al, 1996). An autosomal recessive mutation in murine Fas receptor gives rise to *lpr* (lymphoproliferation) phenotype associated with massive lymphadenopathy and lupus-like autoimmunity. Fas gene mutations have also been found in human beings in an inherited lymphoproliferative disorder associated with autoimmunity, called Canale-Smith syndrome (Drappa, et al,1996). Fas ligand (FasL) is a highly conserved, 40 KDa type II transmembrane glycoprotein that occurs in both membrane-bound and soluble forms (Takahashi et al, 1994 and Tanaka et al, 1995). In mice harbouring the autosomal recessive mutation *gld/gld* (generalized lymphoproliferative disease), a one amino acid residue substitution at position

273 (Phe to Leu) of FasL protein, has also resulted in the development of lymphoproliferation and autoimmunity (Lynch et al, 1994 and Nagata and Suda, 1995). FasL is expressed on many cell types including effector cells of cell-mediated cytotoxicity, i.e. CD4+ and CD8+ T lymphocytes (Hanabuchi et al, 1994), NK cells and LAK cells (Lee et al, 1996 and Arase et al, 1995). On the basis of early studies with alloimmunized CD8+ peritoneal exudate lymphocytes (PEL-CTLs) which were perforin/granzyme deficient, it was first proposed that a nonsecretory CTL-induced triggering of apoptosis can take place, through crosslinking of certain surface molecules like Fas (Shi et al, 1994). The involvement of Fas in cell-mediated cytotoxicity was further supported by the finding that cells from *lpr* mice were refractory to lysis triggered by anti-Fas antibody and by perforin-free CTLs. Furthermore, purified FasL and COS cells transfected with FasL, exhibited cytocidal activity against Fas-bearing cells, while Fas-Fc hybrid molecules and Fas antibody blocked it (Henkart, 1994). Activated T cells from *gld* mice were shown not to be able to lyse Fas positive targets in Ca^{++}-free medium where the perforin/granzyme pathway would be blocked. Now, it is clear that CTLs, NK and LAK cells express FasL, the interaction of which with clustered Fas molecules on a target could bring together the FADD and, thus, initiate intracellular signals that culminate in

apoptotic cell death in the target (Berke, 1995).

4.2. Perforin/Granzyme Pathway

Another effector mechanism employed by immune-derived killer cells is strictly dependent on the presence of extracellular Ca^{++} and involves perforin (pore-forming protein) and granzymes (granule associated enzymes) that are contained within cytoplasmic granules of effector cells and are delivered to the target cell via a process of vectorial exocytosis after adhesion (Henkart and Henkart, 1982). One of the granule proteins, perforin (PFN), also known as cytolysin, was indeed cytolytic in its own right and for a while was believed to be sufficient for death. However, it was then realized that PFN alone was capable only of inducing necrosis (Duke et al, 1989). In contrast, intact killer cells or purified granules were able to cause apoptosis in the target, involving DNA fragmentation. The proteins responsible for this DNA fragmenting ability were ultimately purified and shown to be identical to granzymes (Shi et al, 1992). Granzymes (Grzs) have also been referred to as cytotoxic cell proteases (CCP), cytotoxic T lymphocyte antigen (CTLA), T cell serine proteases (TSP) and cytotoxic serine proteases (CSP). They form part of a multigene family (GrzB to G) that are clustered, close to the α chain of the T cell receptor gene, on chromosome 14 (Crosby et al, 1990). In addition, there is another Grz, referred

to as GrzA or Hannukah Factor (HF) that is encoded in a separate locus (Gershenfeld and Weissman, 1986). Granzymes A and B, the first two Grzs to be described and cloned, have attracted the greatest investigative effort so far. GrzB is the most abundant in cytotoxic lymphocytes, and cleaves proteins to the carboxyl side of acidic amino acids, whereas GrzA possesses trypsin-like specificity, and cleaves adjacent to basic residues (Smyth and Trapani, 1995). GrzB is crucial for rapid induction of apoptosis, while other members of granzyme family elicit a delayed response (Shi et al, 1992 and Shresta S et al, 1997). Recent studies have shown that GrzB induces apoptotic changes in target cells, partly by catalysing the cleavage and activation of several members of ICE/Ced family of proteases, also known as caspases (Cystein-Asp-ases). GrzB also initiates caspase-independent pathways contributing to cell death, although the nature of these additional pathways remains undefined (Andrade et al, 1998). In the most commonly accepted model for the PFN/Grz pathway of immune cytolysis, binding of an appropriate target by a killer cell stimulates a degranulation process in the effector cell. PFN and Grzs, thus released into a closed cleft between the two cells, would then cause target cell lysis. Target cell penetration of the Grzs through polyperforin pores produced in the target cell membrane is thought to trigger signalling pathways leading to apoptosis and the

internal disintegration of the target cell. Extracellular Ca^{++} is essential for exocytosis of granules and insertion and polymerization of PFN on the target cell membrane (Berke, 1995b). Alternatively, GrzB is capable of entering the target independently of PFN. However, apoptosis will not occur until the target is treated with PFN, which appears to act by influencing the intracellular localization of the proteinase (Froelich et al, 1998).

5. Immunotherapy for Cancer

Immunotherapy of cancer attempts to destroy tumor cells using manipulations of the immune system to overcome the poor immune responses elicited by tumors. Several methods have been used to increase MHC expression on tumor cells, including chemical coupling, enzyme treatment, infection of tumor cell lines with vaccinia virus, and introduction of IFN-γ and other cytokine genes into tumor cells to upregulate the expression of self MHC molecules. In murine tumor models, it is now generally accepted that defects in immune regulation, not the absence of tumor antigens, result in failure to mount an antitumor immune response. Investigators are now striving to modify the local tumor cell immunological environment to either enhance the presentation of tumor-specific antigens or to activate tumor infiltrating lymphocytes (Roth et al, 1994). Monoclonal antibodies raised against certain tumor antigens have been

employed to detect metastatic lesions and /or to facilitate tumor destruction by directed chemotherapy or radiotherapy. *In vitro* stimulation of the patient's lymphocytes has emerged as a therapeutic approach in recent years (James, 1997). This approach, however, has been less successful than was first hoped. Adenocarcinomas, in particular, are poorly responsive to adoptive immunotherapy. One reason for this is the inherent ability of many solid tumors to resist attack by cells of the immune system. If this resistance could be overcome, the efficacy of adoptive immunotherapy could be greatly enhanced (MacKenzie et al, 1994).

6. Antiestrogens

Tamoxifen (TX) and toremifene (TO) are two nonsteroidal antiestrogens belonging to triphenylethylene class of compounds that have been or are being used for treatment of breast cancer and endometrial, prostatic and renal cell carcinomas. They are also under investigation for the treatment of other tumors like melanomas and certain leukemias (Baral, *et al*, 1996). Tamoxifen was first noted to modulate the resistance of P388/ADR, a doxorubicin-resistant murine leukemia cell line (Hu, *et* al, 1991). Antiestrogen activity has traditionally been attributed to its ability to bind to and translocate the classical estrogen receptor (ER-α) to the nucleus, where presumably it inhibits estrogen-mediated events

leading to cell growth (Guillot, *et al*, 1996). Contrary to expectations, however, responses have been observed in patients with both ER-negative and -positive tumors, suggesting involvement of alternative mechanisms not directly dependent on tumor ERs (Kim, *et al*, 1990). Antiestrogens are able to directly interact with the transcriptional activation of responsive genes. This is presently the accepted interpretation of the multiple effects of TX and other antiestrogens, these being both cytostatic and cytotoxic to a variety of tumors which need not be hormone-dependent or -responsive (Kellen, *et al*, 1996). It has been shown that TX antagonizes phospholipid- and calmodulin-activated protein Kinase C (Berman, *et al*, 1991). Tamoxifen is useful at or below 10^{-6} M concentration. Above this concentration, TX exerts lethal or sublethal effects; the lethal effects lead to cell death while the sublethal effects may result in cellular transformation and response modification participating in the process of tumor resistance or even tumor stimulation (Jain, *et al*, 1997). Toremifene (TO) is a chlorinated derivative of TX, which is being studied in postmenopausal women for the treatment of advanced cancer. Toremifene was developed to increase the therapeutic-to-toxic ratio of antiestrogens. Like TX, TO has both antiestrogenic and estrogenic properties. Also, as is true for TX, TO binds with a high affinity to cytoplasmic ERs (Stenbygaard , *et al*, 1993 and Jordan, 1995).

7. Antiestrogens and the Immune System

It is established that estrogens and antiestrogens can modulate immune responses both *in vivo* and *in vitro*. Besides, TX and TO affect the neuroendocrine system including the secretion of prolactin and of steroid hormones which are known to regulate immune reactions (Nagy, *et al*, 1997). There is evidence indicating that there are significant differences between the immune effects of various antiestrogenic agents (Baral, *et al*, 1996a).

At therapeutic concentrations, TX exerted an antiproliferative effect on lymphocytes (Baral, *et al*, 1989). Tamoxifen inhibited the expression of C3 complement receptors by human peripheral B cells (Baral, *et al*, 1985). Mitogen-induced proliferation, IL-2 production and IL-2 receptor-α expression by human peripheral blood lymphocytes were all suppressed upon *in vitro* treatment with TX, TO or ICI 164 384 (Teodorczyk-Injeyan *et al*, 1993). Both TX and TO exerted a stimulatory effect on overall cytokine production by a human B cell line (BALL). Tamoxifen also stimulated IL-1β, IL-6 and IFN-γ production by a human T cell line (Molt-4), whereas TO was inhibitory; the more mature T cell line, Jurkat did not show any response. Antiestrogens did not have any significant effect on cytokine production of myeloid cells (Järvinen, et al, 1996). Antiestrogens enhanced the production of TNF-α by LPS-stimulated normal

human monocytes (Teodorczyk-Injeyan, *et al*, 1993).

Estradiol augmented significantly the antigen specific antibody response *in vitro* which was due to the inhibition of suppression by CD8+ T cells and preincubation of the cells with TX prevented this augmentation (Clerici, *et al*, 1991). Treatment of rats with TX (6 mg/Kg) during immunization with sheep red blood cells significantly inhibited the antibody response, which could be reversed by an additional treatment with either prolactin or growth hormone (Nagy and Berczi, 1986).

Contact sensitivity skin reactions were inhibited by TX treatment in rats, and could be restored by an additional treatment with either prolactin or growth hormone. (Nagy and Berczi, 1986). Mixed lymphocyte reaction using rat splenocytes was suppressed by TX *in vitro* (Baral, *et al*, 1991). Antiestrogens are also capable of modulating cell-mediated cytotoxicity which will be discussed later.

II. Specific Background

1. Antiestrogens and Immune Cytolysis - Review of Literature

Several *in vivo* and ex vivo studies using various target/effector systems have demonstrated that antiestrogens are capable of modulating killer cell-mediated cytotoxicity against tumor targets.

Screpanti and coworkers observed augmented murine NK activity against Yac-1 targets and increased splenic LGL number in low density fractions of percoll discontinuous density gradients after a 30-day treatment with either 17 beta-estradiol or the antiestrogen TX. The cytotoxicity was totally abrogated by means of an antiserum to asialo GM1 plus complement, whereas anti-Thy 1.2 antibody treatment only partially diminished the reactivity (Screpanti et al, 1987). Albertini *et al* studied the effects of TX, estradiol or the combination of both on the susceptibility of estrogen receptor (ER)-positive and -negative human breast cell lines to lysis by LAK cells. Both ER^+ (MCF-7) and ER^- (MDA-MB-231) cell lines remained sensitive to lysis by LAK cells and to ADCC after treatment with TX. Both TX (alone)- and estradiol + TX-treated MCF-7 cells were more susceptible than were control MCF-7 cells to lysis by in vitro-generated LAK cells. A similar analysis for MDA-MB-231 cells , however, revealed no change in terms of susceptibility to LAK-induced cytolysis following treatment with estradiol or TX. Moreover, TX treatment made neither MCF-7 nor MDA-MB-231 cells more sensitive to in vivo generated LAK cells obtained from peripheral blood of patients receiving IL-2 without additional in vitro stimulation (Albertini et al, 1992). Numerous papers published by Berczi's group from the University of Manitoba indicate that antiestrogens can boost cell-

mediated cytotoxic reactions in various systems.

Immune cytolysis induced by CTLs raised in cultures of rat spleen cells against Nb2 rat lymphoma cells or conconavalin-A-stimulated rat lymphoblasts was greatly amplified by target cell pretreatment with TX, estradiol or both. More interestingly, the inhibition of DNA or protein synthesis in target cells interfered with both basal cytolysis and drug-induced enhancement, suggesting the requirement for target cell participation in this context. In addition, the release of the nuclear label [3H]-thymidine, from Nb2 targets after CTL cytotoxic insult was enhanced by target treatment with TX (Baral et al, 1994). Similarly, pretreatment of NK-sensitive Yac-1 murine lymphoma cells with TX led to highly significant enhancement of NK cell-mediated cytotoxicity. Besides, treatment of the target cells with the metabolic inhibitors actinomycin D or cycloheximide inhibited the lysis of untreated and TX-exposed cells, and the NK cell-induced nuclear degradation was augmented by TX; target cell sensitization was not mediated by classical ER-α since cytosolic estradiol receptors were not detected in Yac-1 cells (Baral et al, 1995). In another study, treatment of NK-resistant, LAK-sensitive P815 murine mastocytoma cells with either TX or estradiol rendered them more susceptible to LAK cell-induced cytolysis and similar results with the above-mentioned inhibitors were obtained. P815 cells

also lacked classical receptors for estradiol (Baral et al, 1996b). Antiestrogens have also been reported to augment *in vivo* immunotherapeutic effects of immune-derived killer cells in tumor-bearing animals. In an experimental model, enhanced tumor suppression occurred when TX- or TO-treated P815 cells were mixed with *in vitro* generated syngeneic LAK cells prior to subcutaneous injection into normal DBA2 recipients. Tumor suppression could be further increased by treating such recipients orally with TX or TO and by repeated injections of LAK cells into the tumor site. The treatment of mice bearing tumors (5 mm in diameter) orally with TX or TO or intraperitoneally with LAK cells resulted in tumor suppression. When the drug treatment was combined with LAK cells, tumor suppression was more pronounced, and complete tumor suppression was induced in a significant number of animals (Baral E, 1996c).In a similar setup, both TX and TO treatment sensitized P815 cells to lysis by CTLs isolated from the spleens of tumor bearing syngeneic mice. Besides, the transfer of killer cells from tumor hosts resulted in tumor suppression in tumor recipients, which could be enhanced by additional oral treatment with TX or TO. Importantly, complete cure was achieved in a significant number of animals that also showed partial or complete resistence to a subsequent lethal dose of P815 cells (Nagy et al, 1997). We recently found in the human system that

antiestrogens affect both pathways of killer cell-mediated cytotoxicity. Ovarian carcinoma cells recovered from ascitic fluids of cancer patients could be sensitized by TX or TO towards enhanced cytolysis triggered by autologous cytotoxic lymphocytes (Haeryfar et al, 1999a,b). Finally, based on studies in NK-competent athymic nude mice and NK-deficient NIH-3 beige/nude mice, Arteaga and coworkers recently suggested that host NK function might partly mediate the antitumor effect of TX and that TGF-β2 may abrogate this mechanism, thus contributing to TX resistance in human breast carcinomas (Arteaga et al, 1999).

2. K562 Cell Line

The K562 erythroleukemia cell line was originally established from a pleural effusion of a 53-year-old female patient with chronic myelogenous leukemia (CML) in terminal blastic crisis. The K562 cell is typically an undifferentiated blast cell with a diameter of about 20 µm, two or more prominent nucleoli , basophilic cytoplasm containing no granules and a mean doubling time of 12 hours. The cells possess no surface immunoglobulins, Epstein-Barr virus (EBV)-associated antigens, nor do they form rosettes with sheep red blood cells. However, over 90 % of the cells do form EA rosettes, indicating the presence of Fc receptors. The cell membrane glycoproteins exhibit many similarities to

those of red blood cells. Glycophorin A which is found exclusively in human erythrocytes is synthesized by K562. Besides, some of the cells become benzidine positive in the presence of sodium butyrate, and haemoglobin can be detected in K562 by radioimmunoassay. The K562 cells may be induced to produce fetal and embryonic haemoglobin in the presence of 0.1 mM hemin. The presence of the Philadelphia chromosome demonstrates that this cell line is of CML origin (Lozzio and Lozzio, 1975 and Koeffler and Golde, 1980). The absence of T or B lymphocyte markers is an indication that K562 cells are not precursors of lymphopoietic cells (Lozzio et al, 1981). The undifferentiated cells are devoid of alkaline phosphatase and peroxidase activity and lack the ability to ingest inert particles, a property of more differentiated granulocytic cells and macrophages (Lozzio and Lozzio, 1975). That normal human peripheral blood lymphocytes are naturally cytotoxic for K562 cells was first noticed in 1979 (Lozzio and Lozzio, 1979). Today, it is well known that K562 cells comprise the most sensitive target in human natural killer cell-mediated cytotoxicity (Roger R et al, 1996).

3. Antiestrogens and NK-CMC against K562

Mandeville *et al* first suggested that the antiestrogenic agent TX might augment cytotoxicity of healthy donors' peripheral blood lymphocytes as a source of NK

cells against K562 tumor cells. However, it was not clear from the short paper whether target cells, effector cells or both were affected by the drug since TX was just added to the assay (Mandeville et al, 1984). Besides, it was not known at the time that the K562 cells do not bear classical estrogen receptors (Danel et al, 1985). In another study, blood lymphocytes from 23 breast cancer patients who had been treated with TX for 1.5 - 2 years exhibited low NK activity against K562 as compared with those of control patients (Rotstein et al, 1988). Brenner and Margolese monitored the peripheral blood NK activity against K562 in patients with breast cancer receiving no treatment, combination chemotherapy, and/or endocrine therapy. NK activity in untreated stage I patients with no evidence of disease was significantly higher than in healthy controls. Moreover, patients on phenylalanine mustard / doxorubicin / 5-fluorouracil / tamoxifen (PAFT) protocols showed reduced NK activity relative to stage I patients (Brenner and Margolese, 1991).

III. Rationale and Specific Aims

Numerous *in vivo* and *in vitro* studies using various human, murine and rat target/effector systems have shown that antiestrogenic drugs tamoxifen (TX) and toremifene (TO) are capable of augmenting cell-mediated cytotoxic reactions. TX and TO have been used for treatment of several kinds of cancer, particularly

breast cancer. However, the detailed mechanism(s) by which antiestrogens amplify killer cell-mediated tumor destruction has not been fully understood as yet. Having considered preliminary reports indicating that the lymphocytotoxicity against the K562 human erythroleukemia cell line may be enhanced by antiestrogens, we attempted to decipher effects, if any, of antiestrogens on the pathways of immune oncolysis employed by cytotoxic lymphocytes. Our specific aims were: I. to study the role of the Fas/Fas ligand pathway of programmed cell death in antiestrogenic augmentation of NK-cell mediated cytolysis. II. to investigate the effects of antiestrogens on the perforin/granzyme pathway of immune cytolysis.

Materials and Methods

1. Cell Populations

1.1. Cell lines

The K562 human erythroleukemia cell line was grown in RPMI (Roswell Park Memorial Institute)-1640 culture medium, supplemented with 5 μg/ml insulin, 500 μM sodium pyruvate, 50 IU/ml penicillin, 50 μg/ml streptomycin, and 10 % fetal calf serum (FCS)(GIBCO, Burlington, Ontario, Canada). YT-INDY, NK3.3, NKL, Jurkat, Molt-4 Daudi, Raji, P815, Yac-1 and SL2-5 cell lines were all grown in RPMI-1640 complete medium as well. The medium for culturing NK3.3 and NKL was supplemented with 100 IU/ml rhIL-2. All cultures were maintained in humidified atmosphere at 37°C and 5 % CO_2. YT-INDY and NK3.3 cell lines were kindly provided by Dr. Arnold Greenberg. NKL was a gift from Dr. David Frank of Dana Farber Cancer Institute at Harvard University. YT-INDY, NK3.3 and NKL cell lines were used as effector cells in a number of experiments. YT-INDY is a human NK-like cell line which was established from the original line YT (Montel et al, 1995a). YT-INDY is IL-2 independent, utilizes both Fas/FasL and perforin/granzyme pathways, and destroys target cells in a non-MHC-restricted fashion (Montel et al, 1995b). NK3.3 is a human NK cell line derived from a primary mixed lymphocyte culture (MLC). These cells are strikingly similar to the LGL population harbouring the bulk of natural killer

cell activity and depend strictly on IL-2 for growth (Kornbluth et al, 1982).The NKL cell line was first established from the peripheral blood of a patient with CD3- CD16+ CD56+ large granular lymphocyte leukemia. NKL cells resemble normal activated NK cells and are able to mediate natural killing and ADCC. They are also strictly dependent on IL-2 for sustained growth and die if deprived of IL-2 for more than a week (Robertson et al, 1996).

Jurkat is an NK-sensitive, Fas+ human T lymphoma cell line routinely used to detect Fas-mediated cytotoxicity. Jurkat cells are also susceptible to perforin-mediated lysis (Dao et al, 1998).Molt-4 is another NK-sensitive human T lymphoma cell line which abundantly expresses the Fas receptor on its surface (Frederick et al, 1997 and Oshimi et al, 1996).

Daudi and Raji are two NK-resistant human B lymphoma cell lines (Carayol et al, 1998 and Rodella et al, 1998); Daudi is LAK-sensitive and Fas-negative (Stohl et al, 1997 and Frederick et al, 1996), whereas Raji does express the Fas receptor (Rodella et al, 1998).

P815 is an NK-resistant mastocytoma derived from a DBA/2 mouse tumor, which is often used as a suitable target for LAK-mediated cytotoxicity (Dunn and Potter, 1957 and Baral et al, 1996b). P815 cells express only low levels of Fas on their surface (Williams et al, 1997). Yac-1 is a lymphoma which was

originally induced by inoculation of the Moloney Leukemia Virus (MLV) into a newborn A/Sn mouse. This cell line is Fas+ and sensitive to the cytotoxic activity of murine NK cells and is, thus, very often used as a target in NK assays (Kiessling et al, 1975 and Martiniello et al, 1997). SL2-5 is also an NK- and granule extract-sensitive murine lymphoma (Chow, 1991) which can serve as a target in cell-mediated cytotoxicity (CMC).

1.2. Separation of Human Peripheral Blood Lymphocytes (PBLs)

Heparinized (10-15 u/ml) blood samples were obtained from normal volunteers who were in apparent good health and took no medication. Peripheral blood mononuclear cells (PBMCs) as a source of NK cells were isolated by Ficoll-Paque (Pharmacia Biotech, Uppsala, Sweden) density centrifugation at 400 × g for 30 minutes at room temperature. PBMCs were then incubated on plastic dishes for 1 hour at 37°C to deplete monocytes (Taniguchi et al, 1977).

1.3. Preparation of Murine Splenocytes

Spleens were aseptically removed from sacrificed C57BL/6 mice and gently teased. The cells were then suspended in RPMI-1640 complete medium. Large debris was allowed to settle out at room temperature for 5 minutes. Lymphocytes were obtained after centrifugation on Ficoll-Paque gradients at 500 × g for 20 minutes. Then, 2×10^7 cells/ml were washed twice in RPMI-10 % FCS and

depleted of monocytes by incubation in plastic dishes for 1 hour at 37°C in a 5 % CO_2 atmosphere. The non-adherent lymphocytes were harvested, washed, and counted before being used (Carbone et al, 1991).

1.4. *In Vitro* Generation of Lymphokine-activated Killer (LAK) Cells

PBLs (2×10^6/ml) were cultured in RPMI-1640 complete medium supplemented with 100 IU/ml or 500 IU/ml recombinant human interleukin-2 (rhIL-2) for 1 or 5 days at 37°C and 5 % CO_2 to generate activated NK and LAK cells, respectively. Cells were washed and used as effectors in cytotoxicity experiments (Baral et al, 1996b).

2. Drugs and Reagents

2.1. Antiestrogenic Drugs

Tamoxifen (TX) (code ICI-47699) was kindly provided by ICI Pharmaceuticals, Macclesfield, UK and toremifene (TO) (code FC-1157a) was a gift from Orion-Farmos, Finland. Both drugs were initially dissolved in absolute ethanol (10^{-2}M) and appropriately diluted in tissue culture medium. The final concentration of ethanol was less than 0.1 % in the cultures and had no effect on lymphocyte reactions (Baral et al, 1995). Target, effector, or both cell populations were pretreated with TX (1 μM) or TO (5 μM) overnight. Final drug concentrations were chosen based on the highest clinically achievable doses (Jordan, 1982).

2.2. Interleukin-2 (IL-2)

Recombinant human IL-2 (rhIL-2) was obtained from ICN (Costa Mesa, CA). The specific activity of IL-2 was approximately 3×10^6 Biological Response Modifiers Program (BRMP) units per milligram of protein. One BRMP unit of IL-2 is equal to 2.25 international units. The activity was determined by the manufacturer as half-maximal proliferation of IL-2 dependent CTLL-2 cells.

2.3. Antibodies

Anti-human Fas monoclonal antibody, CH-11 is an affinity- purified antibody of IgM class obtained from Balb/c mouse ascites following immunization with human diploid fibroblast cell line FS-7. It reacts specifically with human Fas receptor and does not exhibit any cross-reaction with mouse Fas receptor or other members of TNF receptor family. One μg/ml of the antibody was used instead of effector cells to induce apoptosis in a number of cytotoxicity experiments (Weller et al, 1994).

According to the supplier, anti-mouse Fas monoclonal antibody, RK8 is an IgG fraction purified by ammonium sulfate precipitation and protein G chromatography after immunization of Arminian hamsters with recombinant chimera protein of soluble mouse Fas and AIC2A. RK8 reacts specifically with the Balb/c and MRL mouse Fas receptor and induces apoptosis. It also weakly

reacts with C3H mouse Fas antigen. In order to induce apoptosis in target cells in question, RK8 was used at a final concentration of 100 ng/ml.

In flow cytometric analyses, anti-CD95 (Fas/Apo-1) FITC-conjugated mAb was used along with IgG_1-FITC isotype control antibody to detect surface Fas expression. All antibodies mentioned above were purchased from Kamiya Biomedical Company, Seattle, WA.

2.4. Inhibitors

Benzyloxycarbonyl-Val-Ala-Asp (beta-O-methyl)- fluoromethylketone (z-VAD-fmk) and benzyloxycarbonyl-Ala-Ala-Asp-chloromethylketone (z-AAD-cmk) were purchased from Kamiya Biomedical Company, Seattle, WA. Z-VAD-fmk is a broad spectrum caspase inhibitor (Vercammen et al, 1998) which was initially dissolved in dimethyl sulfoxide (DMSO) and used at a final concentration of 100 μM for overnight pretreatment of target cells and was also present during the cytotoxicity assay. According to the supplier, fmk-type inhibitors are synthesized as methyl esters and thus easily enter intact cells, even at low concentrations, without cytotoxic effects. The methyl groups are removed by esterases *in vivo*. Z-AAD-cmk is a peptide inhibitor which specifically inhibits both human and mouse granzyme B (Okake et al, 1991). However, overnight pretreatment of PBLs with 100 μM z-AAD-cmk turned out to be

highly cytotoxic for the cells in our experiments. Besides, cmk inhibitors penetrate cells poorly (author's personal communications with Dr. Arnold Greenberg).

The general mechanism-based serine protease inhibitor DCI (3,4-Dichloroisocoumarin) was a generous gift from Sigma-Aldrich Canada, Ltd. DCI which is often used to inhibit granzymes in intact cells (Vermijlen et al, 1999) releases a reactive acyl moiety which can then acylate yet another active site in the enzyme to be inhibited (Harper et al, 1985). PBLs were pretreated for 1 hour with DCI at a final concentration of 50 μM before being used in some cytotoxicity experiments (Roger et al, 1996).

3. ^{51}Cr Release Cytotoxicity Assay

Specific ^{51}Cr (Amersham, Oakville, Canada; specific activity: 250-500 mCi/mg Cr; 9.25-18.5 giga Becquerel/mg Cr) release from target cells was determined after a 5-hour incubation in triplicate at 37 °C and 5 % CO_2 (Baral et al, 1996b). Target cells were labelled with ^{51}Cr (100 $\mu Ci/10^7$ cells) for one hour, washed, and 10^4 cells/100 μl were plated into each well of U-bottomed microtiter plates. Effector cells were added in 100 μl at the indicated ratios and incubated for 5 hours. After incubation, 100 μl of supernatant were harvested from each well and counted with a gamma counter (Universal Gamma Counter, 1282

Compugamma CS). The percentage of specific release was then calculated according to the formula:

$$\text{Percent Cytotoxicity} = (ER - SR / TR - SR) \times 100$$

where ER stands for experimental release. Total release (TR) was obtained from wells receiving 1 % Triton X-100. Spontaneous release (SR) obtained from wells in which radiolabelled target cells alone were incubated with 100 µl of medium instead of effector cells, was always less than 10 % of TR. Mean cpm ± SEM (counts per minute ± standard error of the mean) was calculated and used for the reporting of the data.

4. Calcium Chelation by EGTA/$MgCl_2$

In a number of experiments, extracellular Ca^{++} was chelated by EGTA [Ethylene glycol-bis-(β-aminoethyl ether)N,N'-tetraacetic acid] and $MgCl_2$ which were added to the assay at final concentrations of 1 mM and 1.5 mM, respectively (Roger et al, 1996).

5. Flow cytometry Analysis of Fas Receptor

Aliquots of 10^6 K562 tumor cells were washed with Hank's balanced salt solution (HBSS) supplemented with 5 % FCS and 0.02 M HEPES (pH 7.2). One

μg of anti-CD95 (Fas/Apo-1) FITC-conjugated monoclonal antibody was added to 100 μl of the cell suspension followed by incubation on ice for 1 hour. Controls were treated with 1 μg of IgG_1-FITC. Cells were then washed twice, fixed with 200 μl of 1 % paraformaldehyde and finally subjected to FACS analysis using a Coulter Epix V machine (Argon Ion Laser Line 488 nm).

6. Statistical Analysis

Student's t-test was used for statistical analyses of data and p values less than 0.05 were considered significant.

Results

I. Antiestrogens And Natural Killer Cell-mediated Destruction of K562

1. The Effect of Antiestrogenic Pretreatment Alone on NK-CMC

In order to confirm an earlier report indicating an antiestrogenic augmentation of NK cell-mediated lysis of K562 (Mandeville et al, 1984); and to further clarify which cells are affected by antiestrogens; target cells, effector cells, or both were treated with toremifene (5 μM) overnight before being used in ^{51}Cr release assay. The results of 5 independent experiments using freshly isolated PBLs as effectors are represented in Figure 1. The killing was most pronounced when both K562 and effector cells were pretreated with TO. There was also a significant enhancement of cytotoxicity when K562 cells were treated. Pretreatment of PBLs alone, however, resulted only in a trend for enhanced cytolysis (Fig. 1).

The results obtained from two experiments employing YT-INDY NK-like cell line as effector cells are summarized in table 1, according to which both effector and target cells need to be pretreated in order for the maximal cytotoxicity to be achieved. YT-INDY cells lost their cytotoxic capability against K562 after few days of culturing in RPMI complete medium. Similarly in a search for a constant source of NK cells capable of lysing K562, we were not able to detect any cytotoxicity by human NK3.3 and NKL cell lines or murine splenocytes in our

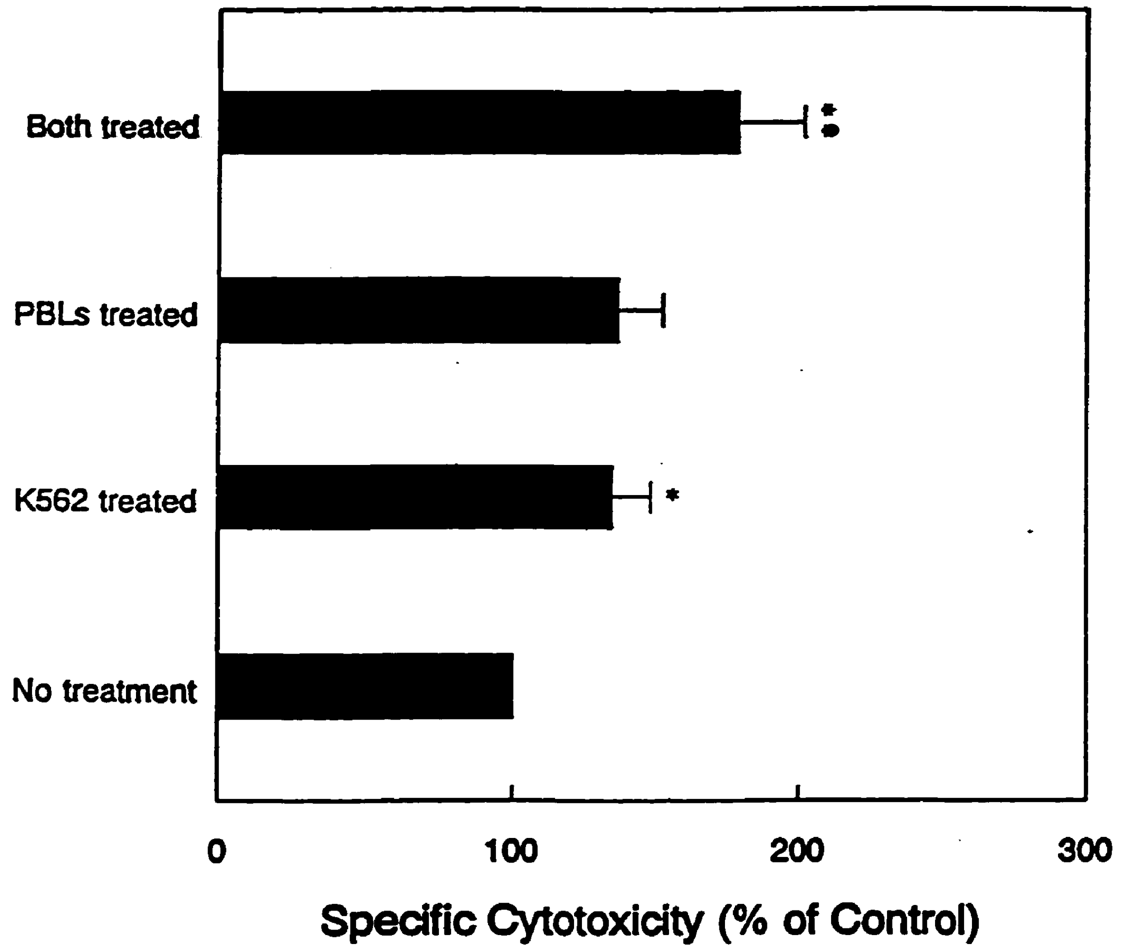

Figure 1. The effect of antiestrogenic pretreatment on NK cell-mediated cytotoxicity against K562 tumor targets. Human peripheral blood lymphocytes obtained from 5 normal volunteers, human erythroleukemia cells or both were treated with toremifene (5 μM) overnight before being used in a standard 5-hour 51-Cr release cytotoxicity assay using an effector:target ratio of 25:1. * $p = 0.05$, ** $p < 0.05$, $n = 5$

^{51}Cr release assays (Table 2).

2. The Effect of Antiestrogens on Anti-Fas Antibody-mediated Lysis of K562

K562 leukemia cells were not killed by anti-human Fas antibody, CH-11 used instead of effector cells in cytotoxicity assays, whereas Fas-expressing Jurkat cells used as positive controls were efficiently lysed under identical conditions. Moreover, treatment with antiestrogens had no influence on the anti-Fas-mediated cytolysis (Table 3).

3. The Effect of Antiestrogens on Fas Receptor Expression by K562

Pretreatment with antiestrogens did not change the expression of Fas receptor (CD95/Apo-1) at the protein level as assessed by FACS analysis (Fig. 2).

4. The Effect of Ca^{++} Chelation on Antiestrogen Modulation of NK-CMC

In order to characterize the mechanism(s) of antiestrogen-induced modulation of NK-CMC against K562, the perforin/granzyme pathway of immune cytolysis was blocked by adding EGTA/$MgCl_2$ to the assays to chelate extracellular Ca^{++} cations which are strictly required for granule-mediated killing (Williams et al, 1997). The lysis of K562 was completely abrogated wherever EGTA/Mg^{++} was present in the assay. Importantly, where antiestrogens exerted an enhancing effect on NK-CMC against K562, this effect was also blocked since the cytotoxicity was reduced to background levels by the presence of EGTA/$MgCl_2$

			% specific 51-Cr release (Mean ± SEM)			
Exp.	E:T ratio	Antiestrogen	NT	TT	ET	BT
1	5:1	TX (1 µM)	10.0±2.7	12.5±4.2	8.9±4.5	23.6±2.7
	5:1	TO (5 µM)	10.0±2.7	15.2±3.0	14.3±2.3	20.7±2.6
2	5:1	TO (5 µM)	7.0±1.1	7.6±0.2	8.2±1.1	12.1±1.5
	10:1	TO (5 µM)	7.0±0.4	10.2±0.7*	10.2±2.2	13.0±1.1

Table 1. The effect of antiestrogenic treatment on YT-INDY-mediated K562 cytolysis. Cells were incubated with medium or indicated antiestrogens before being used in a standard 5-hour 51-Cr release assay. Values represent mean ± SEM in triplicate wells. NT: no treatment; TT: target treated; ET: effector treated; BT: both treated.

Effector	E:T ratio	SR	ER	TR	% specific cytotoxicity
NK3.3	25:1	1293±91	1650±110	19803±253	1.9±0.6
NKL	25:1	1599±21	1445±39	23900±739	0
Splenocytes (mouse)	12.5:1	677±23	733±60	10397±153	0.8±0.7

Table 2. Specific cytotoxicity of human NK3.3 and NKL cell lines and murine splenocytes against K562 in a 5-hour 51-Cr release assay. Values represent mean (± SEM) cpm in triplicate wells. SR: spontaneous release; ER: experimental release; TR: total release (see materials & methods).

Target	SR	ER	TR	% Specific Cytotoxicity
K562	3850±203	3563±350	24606±332	0
K562-TO	4078±34	3892±91	26531±568	0
Jurkat	4290±550	10990±512	25120±1070	32.5±3.7

Table 3. The effect of toremifene pretreatment on K562 cytolysis triggered by anti-Fas antibody. K562 cells were incubated overnight with medium or toremifene (5 μM) prior to a 5-hour 51-Cr release assay using 1 μg/ml of the CH-11 anti-human Fas mAb instead of effector cells. Fas+ Jurkat cell line was used as a positive control. Data are shown as mean (± SEM) cpm in triplicate wells. SR: spontaneous release; ER: experimental release; TR: total release; K562-TO: K562 cells treated with toremifene.

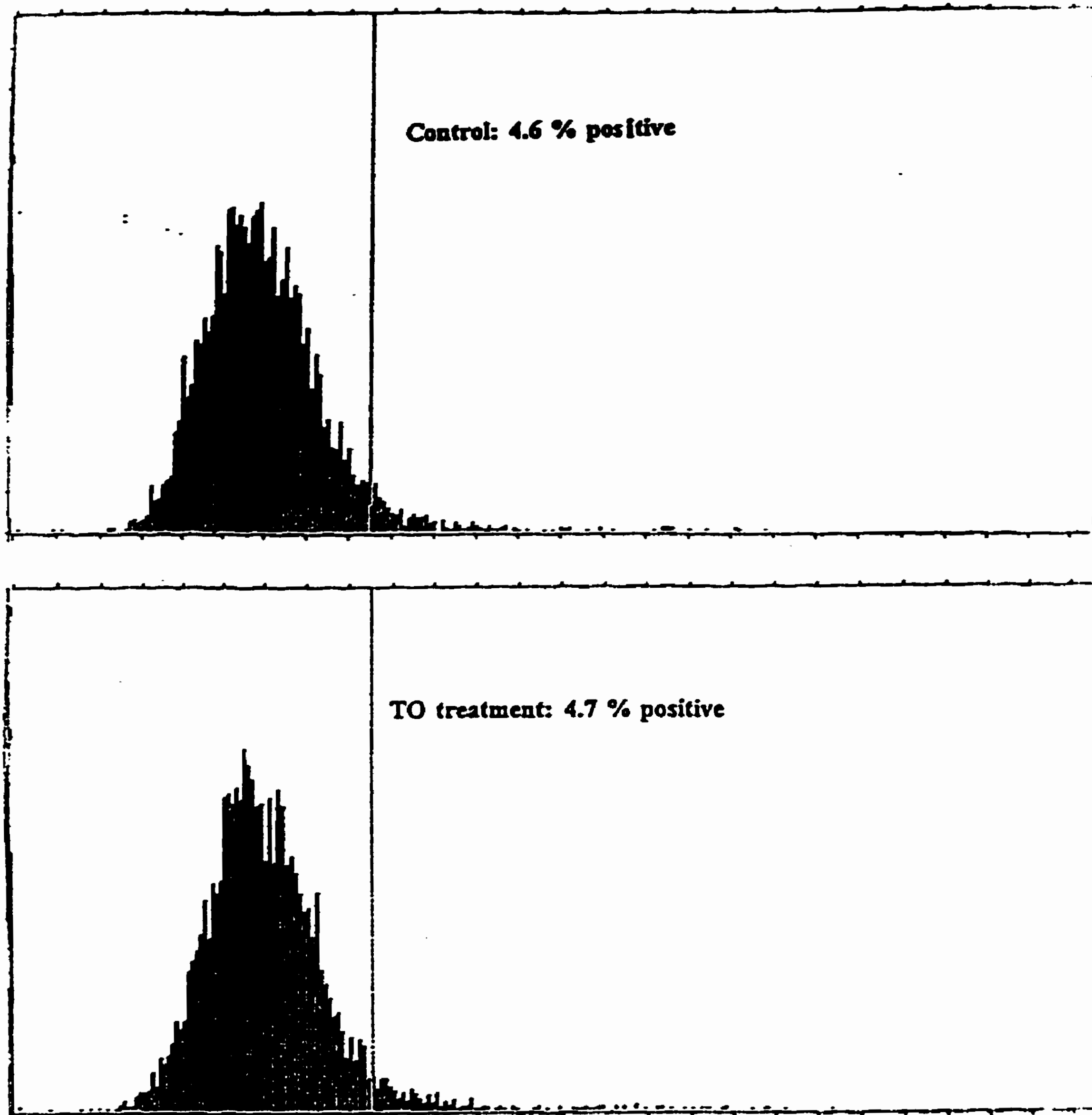

Figure 2. The effect of antiestrogenic pretreatment on Fas receptor (CD95/Apo-1) expression by K562 human erythroleukemia cells. One μg of FITC-conjugated anti-Fas mAb was added to nontreated and TO-pretreated K562 cells followed by FACS analyses. One μg of IgG_1-FITC antibody was also used in the experiment as the isotype control.The data are expressed as the percentage of positive cells.

in the assays (Fig. 3).

5. The Effect of General Caspase Inhibitor z-VAD-fmk on Antiestrogen Modulation of NK-CMC

Broad-spectrum caspase inhibitor z-VAD-fmk used in one cytotoxicity experiment to treat K562 cells did not inhibit NK-CMC, but rather unexpectedly, increased specific chromium release from targets in all groups regardless of antiestrogenic treatment and this increase was statistically significant ($p < 0.05$)(Table 4).

6. The Effect of Serine Protease Inhibitor DCI on Antiestrogen Modulation of NK-CMC

Treatment of PBLs with the general mechanism-based serine protease inhibitor DCI significantly, but not completely inhibited the specific NK-CMC against K562. However, unlike EGTA, DCI failed to reduce the specific cytotoxicity to the background level in the antiestrogen-treated groups (Fig. 4).

II. Antiestrogens and IL-2-activated Killer Cell-mediated Destruction of K562

Overnight incubation of freshly isolated PBLs obtained from normal volunteers with 100 IU/ml of IL-2 resulted in a significant augmentation of K562 cytolysis. Furthermore, a 5-day incubation of PBLs with 500 IU/ml of IL-2 which

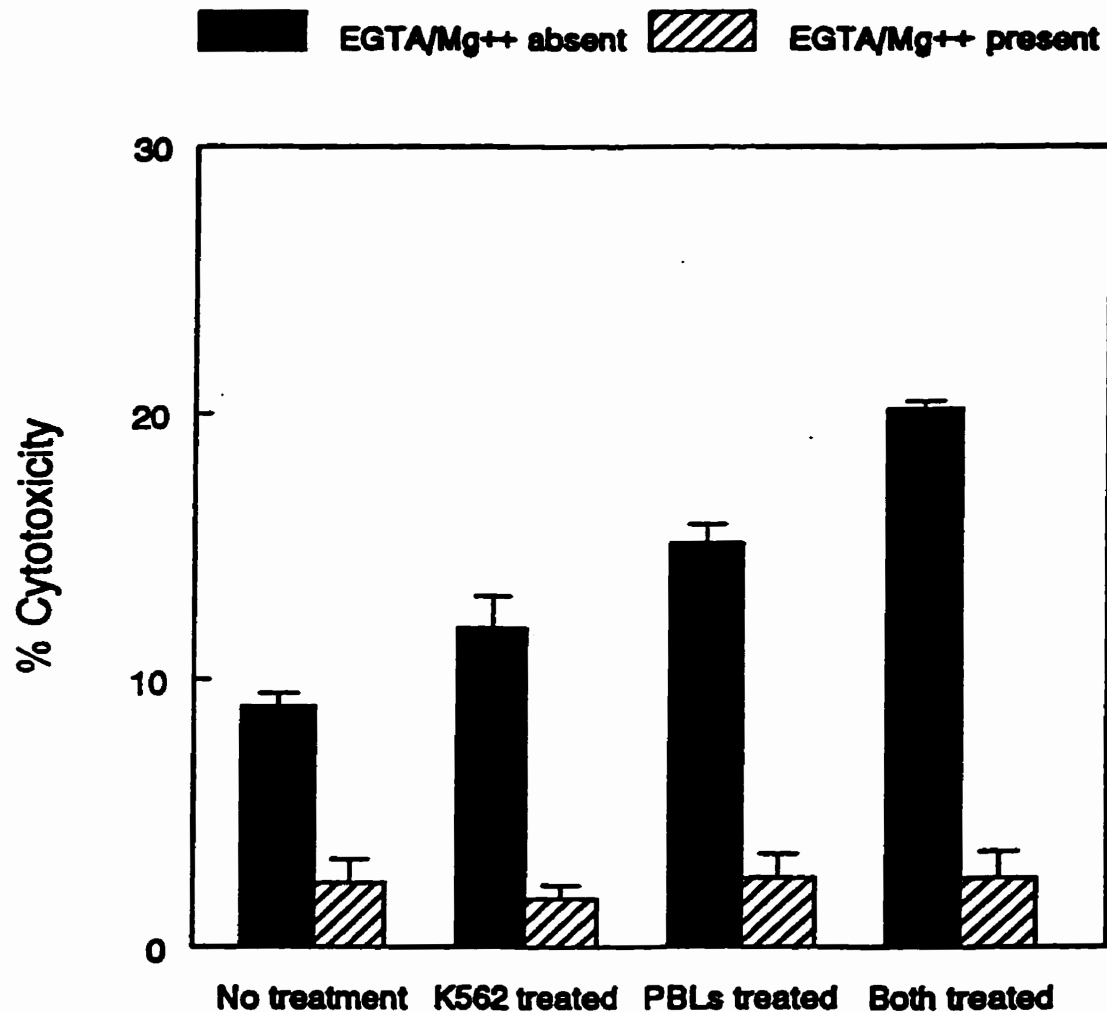

Figure 3. The effect of Ca^{++} chelation on NK cell-mediated K562 cytolysis with or without antiestrogen treatment. Peripheral blood lymphocytes and toremifene-treated and -nontreated K562 tumor cells were used at an effector:target ratio of 25:1 in a standard 5-hour ^{51}Cr release assay in the presence or absence of the Ca++ chelator, EGTA/$MgCl_2$. Results obtained from one experiment representative of 3 independent experiments are shown as mean (± SEM) in triplicate wells.

TO Treatment	zVAD-fmk	% specific lysis
None	-	5.7 ± 0.7
	+	10.0 ± 0.9
K562 treated	-	5.2 ± 0.7
	+	9.1 ± 0.6
PBLs treated	-	6.2 ± 1.2
	+	11.9 ± 0.9
Both treated	-	7.9 ± 0.3*
	+	10.2 ± 0.7

Table 4.The effect of general caspase inhibitor z-VAD-fmk on antiestrogen modulation of K562 immune cytolysis. zVAD-fmk (100 μM) was used for overnight pretreatment of K562 target cells and was also present during a 5-hour 51-Cr release assay. Values represent mean (± SEM) in triplicate wells.

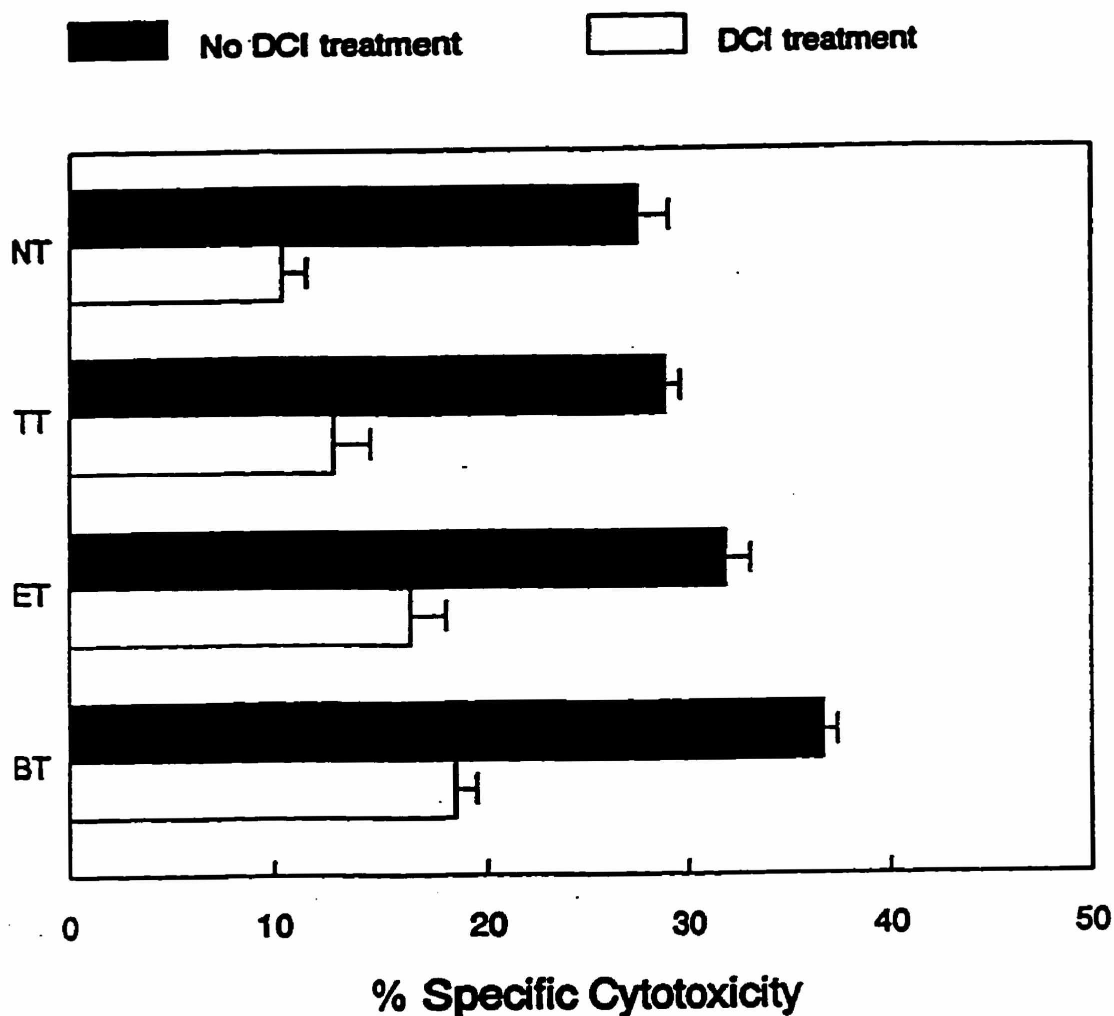

Figure 4. The effect of serine protease inhibitor DCI on NK cell-mediated K562 cytolysis with or without antiestrogen treatment. Peripheral blood lymphocytes were pretreated for 1 hour with 50 μM DCI followed by a further treatment with medium or toremifene (5 μM) before they were used against toremifene-treated or -nontreated K562 at an E:T ratio of 25:1. Results obtained from one experiment are shown as mean (± SEM) specific cytotoxicity in triplicate wells. NT: no treatment; TT: target treated with TO; ET: Effector treated with TO; BT: both target & effector treated with TO.

according to the literature, gives rise to a highly cytotoxic cell population known as LAK (Lymphokine-activated Killer) cells (Baral et al, 1996b) led to a more pronounced elevation of cytotoxicity as expected (Fig. 5). Surprisingly, when either IL-2-stimulated PBLs or LAK cells were used as effector cells against K562, a consistent reduction in their cytotoxic capability was noticed upon treatment with toremifene with an overall inhibition when both target and effector cells were pretreated. Similar to fresh PBLs, IL-2-stimulated killer cells destroyed K562 targets via the perforin/granzyme pathway inasmuch as cytotoxicity was completely blocked in all groups by the presence of EGTA/Mg^{++} in the assay (Table 5 and Figure 6).

III. Antiestrogens and Anti-Fas-mediated Lysis of Other Tumor Targets

Taking into consideration our recent finding indicating that antiestrogens can upregulate the Fas receptor in some, but not all tumor cells (Haeryfar et al, 1999a) and the fact that cytotoxic lymphocytes can utilize the Fas/FasL pathway to destroy their targets, we looked into any possible effect exerted by antiestrogens on anti-Fas-mediated killing of a few other human and murine tumor targets with different characteristics in terms of NK- or LAK-sensitivity and Fas expression (See Table 6 & 7 and also materials and methods for details). CH-11 anti-Fas monoclonal antibody was cytolytic against Jurkat, Molt-4 and

Raji, and not against Daudi cell line, as expected. We could not detect any significant change in specific cytolysis triggered by CH-11 upon antiestrogen treatment of targets (Table 6).

Of three murine targets used (Yac-1, P815 and SL2-5), none was substantially susceptible to cytolysis induced by RK8 anti-mouse Fas antibody and pretreatment of tumor cells with toremifene made no difference in this respect (Table 7).

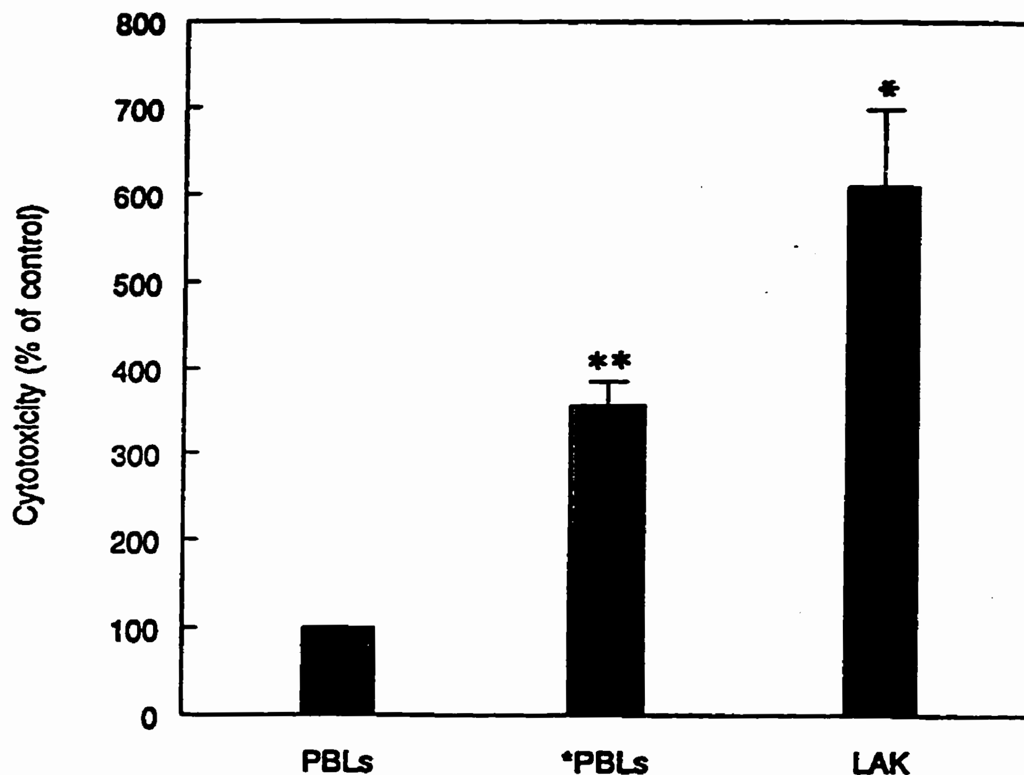

Figure 5. The effect of Interleukin-2 on K562 immune cytolysis. Peripheral blood lymphocytes obtained from 3 normal volunteers were used freshly and after incubation with rhIL-2, against K562 tumor cells at an E:T ratio of 5:1 in a 5-hour 51-Cr release assay. Data are expressed as mean (± SEM) cytotoxicity (% of fresh PBL control). *PBLs: PBLs incubated with 100 IU/ml rhIL-2for 24 hours; LAK: PBLs incubated with 500 IU/ml rhIL-2 for 5 days; * $p < 0.005$; ** $p < 0.001$, $n = 3$. Cytotoxicity of LAK cells was also significantly higher than that of *PBLs ($p = 0.05$).

Exp.	TO	EGTA	Effector	E:T	% specific 51-Cr release (Mean±SEM) NT	TT	ET	BT
1	-	-	*PBLs	5:1	15.2±0.7	-	-	-
	-	+	*PBLs	5:1	1.0±0.6	-	-	-
	+	-	*PBLs	5:1	-	16.1±1.3	12.3±0.4	11.2±0.7
	+	+	*PBLs	5:1	-	0.3±0.1	0.9±0.9	0
	-	-	*PBLs	10:1	27.8±1.5	-	-	-
	-	+	*PBLs	10:1	1.3±0.4	-	-	-
	+	-	*PBLs	10:1	-	28.5±1.0	20.2±1.5	22.0±0.5
	+	+	*PBLs	10:1	-	0.6±0.4	1.2±0.8	1.6±1.4
	-	-	LAK	5:1	19.9±1.1	-	-	-
	-	+	LAK	5:1	1.2±0.1	-	-	-
	+	-	LAK	5:1	-	22.6±1.1	16.6±2.1	24.1±1.5
	+	+	LAK	5:1	-	1.4±0.2	0	3.1±0.1
2	-	-	*PBLs	5:1	7.2±0.8	-	-	-
	-	+	*PBLs	5:1	0.8±0.4	-	-	-
	+	-	*PBLs	5:1	-	7.9±0.9	5.6±0.9	5.8±0.4
	+	+	*PBLs	5:1	-	1.2±0.4	0.2±0.2	0.3±0.2
	-	-	*PBLs	10:1	16.2±0.8	-	-	-
	-	+	*PBLs	10:1	0.6±0.4	-	-	-
	+	-	*PBLs	10:1	-	15.7±0.4	5.4±1.9	12.5±1.4
	+	+	*PBLs	10:1	-	1.0±0.1	0.3±0.2	1.5±0.8
	-	-	LAK	2.5:1	32.8±2.5	-	-	-
	-	+	LAK	2.5:1	2.2±0.3	-	-	-
	+	-	LAK	2.5:1	-	35.7±1.6	25.3±0.7	29.3±1.1
	+	+	LAK	2.5:1	-	2.9±1.6	1.2±0.3	3.7±1.2
	-	-	LAK	5:1	51.5±0.7	-	-	-
	-	+	LAK	5:1	3.0±0.3	-	-	-
	+	-	LAK	5:1	-	54.8±2.0	42.5±2.4	47.1±0.9
	+	+	LAK	5:1	-	4.3±1.2	3.3±0.7	3.5±1.6
3	-	-	LAK	2.5:1	28.1±0.9	-	-	-
	-	+	LAK	2.5:1	1.7±0.9	-	-	-
	+	-	LAK	2.5:1	-	26.7±1.1	15.2±1.0	14.4±0.9
	+	+	LAK	2.5:1	-	3.2±1.0	1.0±0.4	3.4±0.3
	-	-	LAK	5:1	42.9±1.0	-	-	-
	-	+	LAK	5:1	3.4±0.5	-	-	-
	+	-	LAK	5:1	-	45.2±0.9	25.4±0.2	32.5±1.2
	+	+	LAK	5:1	-	4.6±0.9	2.5±0.6	3.3±0.5

Table 5. The effect of Ca^{++} chelation on K562 oncolysis induced by IL-2-stimulated PBLs or LAK cells with or without antiestrogen treatment. NT: no treatment; TT: target treated with TO (5 μM, overnight); ET: effector treated with TO; BT: both target and effector treated with TO; *PBLs: peripheral blood lymphocytes stimulated by rhIL-2 (100 IU/ml, 24 hr); LAK: PBLs stimulated by rhIL-2 (500 IU/ml, 5 days). Results are shown as mean (± SEM) in triplicate wells for each independent experiment.

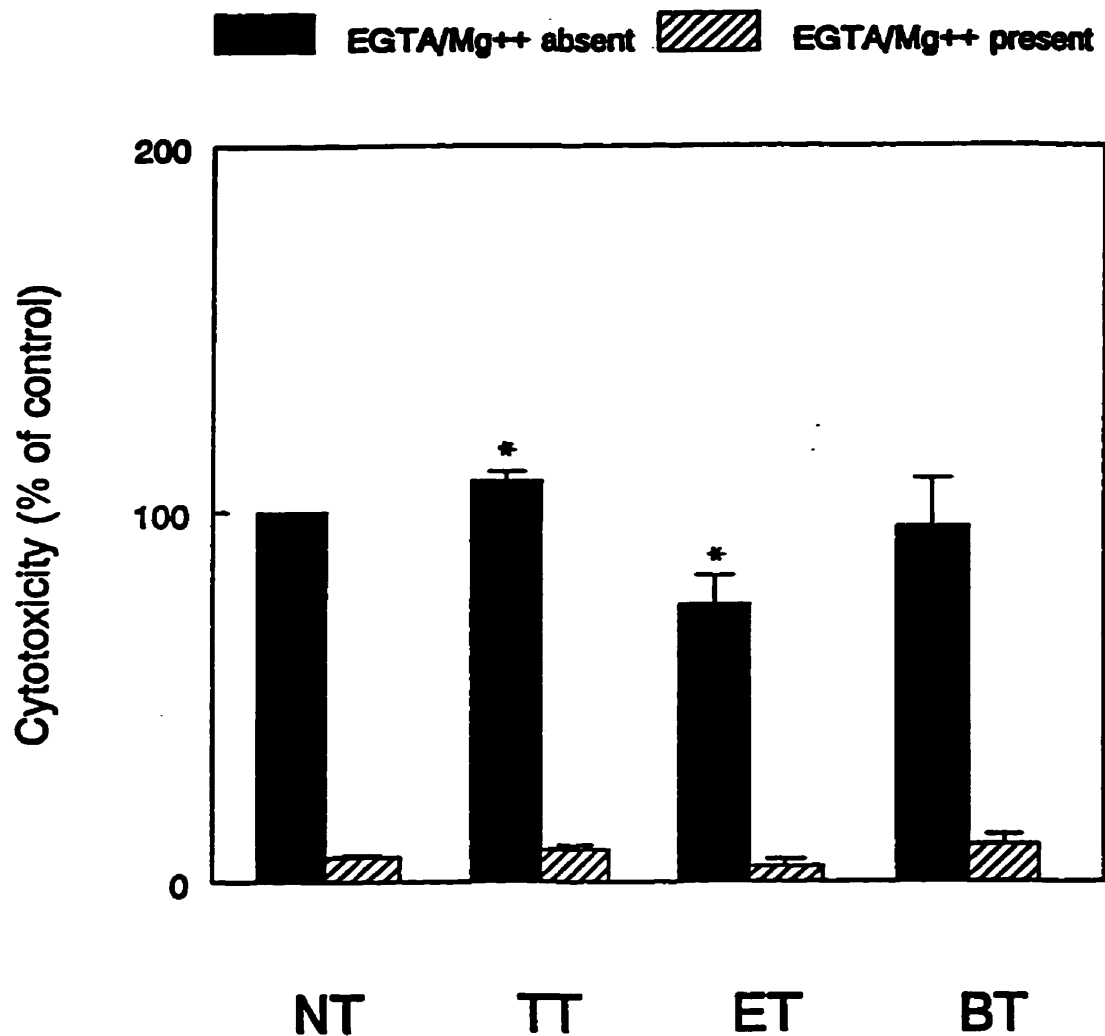

Figure 6. The effect of Ca^{++} chelation on LAK cell-mediated K562 cytolysis with or without antiestrogen treatment. LAK cells generated by incubation for 5 days of PBLs with 500 IU/ml IL-2 were treated with medium or toremifene (5 μM) and used at an E:T ratio of 5:1 against TO-treated or -nontreated K562 in the presence or absence of the Ca^{++} chelator, $EGTA/MgCl_2$. The results of 3 independent experiments are shown as mean (± SEM) cytotoxicity (% of control). * $p < 0.05$

Exp.	Target	CD95	NK sensitivity	LAK sensitivity	treatment	% Lysis
1	Jurkat	+	+	+	None	32.8±3.3
					TX (1μM),4 hr	26.9±1.3
					TX (1μM),24 hr	34.1±2.3
					TO (5μM),4 hr	27.8±5.5
					TO (5μM), 24 hr	31.5±1.7
2	Molt-4	+	+	+	None	24.2±1.2
					TO (5μM), 24 hr	20.6±0.4
3	Daudi	-	-	+	None	0.7±0.5
					TO (5μM), 24 hr	0.2±0.2
4	Raji	+	-		None	7.3±0.3
					TO (5μM), 24 hr	7.4±0.4

Table 6. The effect of antiestrogenic treatment on anti-Fas-mediated cytolysis of other human tumor targets. The CH-11 anti-human Fas mAb was employed in lieu of effector cells in a 5-hour Cr release assay. Data are expressed as mean (± SEM) specific cytotoxicity in triplicate wells. No significant difference was noticed between antiestrogen-treated and control groups.

Target	CD95	NK Sensitivity	LAK Sensitivity	Treatment	% Lysis
Yac-1	+	+	+	None	3.1±2.2
				TO (5μM), 24 hr	2.0±1.4
P815	Low	-	+	None	0.9±0.5
				TO (5μM), 24 hr	0.3±0.3
SL2-5		+	+	None	0
				TO (5μM), 24 hr	1.3±0.9

Table 7. The effect of antiestrogenic treatment on anti-Fas-mediated cytolysis of murine tumor targets. The RK8 anti-mouse Fas mAb was used at 100 ng/ml against the targets. Values represent mean (± SEM) specific lysis in triplicate wells. No significant difference was noticed between antiestrogen-treated and -nontreated groups.

Discussion

I. General Discussion

The idea of treating cancer by immunization has been investigated for over three decades. Although there is evidence from well-defined animal models that immunoprevention and immunotherapy of cancer may be feasible, progress in the treatment of cancer has been by and large slow and disappointing. Killer cells are frequently detectable in cancer patients and tumor bearing animals and may be present in the tumor tissue itself. It has been observed in a number of laboratories that the cytotoxic effector cells removed from tumor bearing hosts are capable of killing the tumor targets in vitro, yet they are incapable of eradicating the same cancer in the host (Berczi et al, 1973). In recent years, considerable attention has been paid to boosting cytotoxic capabilities of immune-derived killer cells as a therapeutic modality to fight cancer. The triphenylethylene-derived antiestrogens tamoxifen and toremifene have been or are being used for treatment of breast cancer, endometrial, prostatic and renal cell carcinomas. They are also under investigation for other tumors like melanomas and certain leukemia (Baral et al, 1996a). Numerous studies using various murine, rat and human target/effector systems have indicated an antiestrogenic augmentation of immune cytolysis. Antiestrogen activity has traditionally been ascribed to its ability to bind to and translocate the classical

estrogen receptor (ER-α) to the nucleus where it presumably inhibits estrogen-mediated events leading to cell growth (Guillot et al, 1996). However, responses have been observed in patients with both ER-negative and ER-positive tumors. Besides, antiestrogenic enhancement of cell-mediated cytotoxic reactions has been observed against both ER-positive and -negative tumor targets. It is clear from earlier studies that the presence of the classical estrogen receptor is not a prerequisite for such an enhancing effect (Haeryfar et al, 1999b). A second estrogen receptor (ER-β) has been described recently, which can bind estradiol and can transactivate estrogen regulated reporter genes, although less efficiently than ER-α, and antiestrogens inhibit this effect. ER-β has an overlapping but non-identical tissue distribution to ER-α (Dotzlaw et al, 1997). It is possible that ER-β also plays a role in the context of enhanced cytolysis by antiestrogens.

Our results show that antiestrogens are able to enhance killing of K562 tumor targets attacked by NK cells. K562 is a human cell line which was first established from the pleural effusion of a 53-year-old female with chronic myelogenous leukemia (CML) in terminal blast crises (Lozzio and Lozzio, 1975). Standard chemotherapy regimens are largely inefficient in CML and despite a demonstration of an increased survival pattern of patients treated with interferon-α versus hydroxyurea, allogeneic bone marrow transplantation during

the chronic phase remains the only curative approach for a large number of patients, this response being related to both the conditioning regimen and the graft-versus-leukemia (GVL) effect. Moreover, donor lymphocyte transfusions which induce a GVL effect have been used successfully for adoptive immunotherapy of CML cases relapsing after bone marrow transplantation (Van Rhee et al, 1994 and Roger et al, 1996). In fact, CML is exquisitely sensitive to immune recognition, and adoptive immunotherapy has become standard clinical practice in the management of CML (Dazzi and Goldman, 1998). Interestingly, in post-transplant CML cases, the risk of relapse is lower in patients whose IL-2-stimulated peripheral blood mononuclear cells display a significant lytic activity *in vitro* against host-derived CML targets, and this cytotoxic activity has been attributed to CD16+ CD56+ cells, but not CD3+ lymphocytes (Hauch et al, 1990). Similarly, K562 cells which have been found to be unusually resistant to apoptosis induced by a variety of agents like irradiation, ultraviolet light, chemical inducers and protein synthesis inhibitors, comprise the most sensitive targets in human natural killer cell-mediated cytotoxicity (Roger et al, 1996). Here, we provide data that antiestrogenic drugs are able to amplify NK cell-induced cytotoxicity against K562 and that such an amplification is most pronounced when both target and effector cells are treated with antiestrogens.

Cytotoxic effector cells derived from the immune system destroy their targets by two main mechanisms namely, Fas/FasL and perforin/granzyme pathways. We looked into the possibility that one or both of these known pathways might be influenced by antiestrogens, resulting in enhanced immune cytolysis of K562.

II. Antiestrogens, Fas/FasL Pathway and K562 Cytolysis

Human natural killer cells comprise about 10-15 % of PBMCs and play a key role in innate immune defences (Silva et al, 1994). Peripheral blood lymphocytes as a source of NK cells can easily kill K562 tumor targets, and K562 cytolysis by PBLs is indicative of NK activity. Although many studies have documented the involvement of the Fas/FasL pathway in CTL-mediated cytotoxicity, the role of the Fas pathway in NK cell-induced lysis has been controversial. Poly(I).Poly(C)-activated NK cells from perforin-deficient mice completely lacked cytotoxic activity against Fas+ Yac-1 cell line (Kagi et al, 1994). On the other hand, Arase et al demonstrated the involvement of Fas-mediated killing in murine NK-CMC (Arase et al, 1995). Montel et al and Tanaka et al also reported that NK-like tumor cells, such as YT-INDY, large granular lymphocytic leukemia and NK lymphoma cells, kill Fas receptor transformant cells via the Fas pathway. Fresh human NK cells were shown to be able to utilize the Fas lytic pathway after stimulation with phorbol-12- myristate-13- acetate/ionomycin

(Montel et al, 1995 and Tanaka et al, 1996). It is also in the literature that NK cells use the Fas/FasL pathway only as an alternative to the perforin/granzyme pathway. FasL expressed on NK cells can induce apoptosis in target cells that express high levels of Fas (Vermijlen et al, 1999). Mori *et al* also provided evidence that the Fas pathway is operative in human PBMC-derived fresh NK cells under certain conditions that are dependent on the nature of the target cells used. It is not known whether surface FasL expression on NK cells is increased following interaction with target cells (Mori et al, 1997). K562 cells do not bear Fas receptor and even transfection with Fas does not make them susceptible to anti-Fas-mediated lysis (Montel et al, 1995). Our cytotoxicity and flow cytometry results confirmed the lack of Fas expression by K562. Moreover, pretreatment with antiestrogens did not change the Fas expression by K562 despite increased NK-mediated cytolysis. Therefore, antiestrogen augmentation of K562 cytolysis cannot be through manipulation of the Fas/FasL pathway. This was further confirmed by our observation that K562 cytolysis and its antiestrogen augmentation were strictly dependent on the presence of extracellular Ca^{++} in the assay. A characteristic of the Fas/FasL pathway is independence of extracellular Ca^{++}. It should, however, be noted that extracellular Ca^{++} ions are necessary for CTL FasL upregulation upon activation,

but once FasL is expressed, the Ca^{++}-independent nature of the Fas cytotoxicity can be clearly demonstrated (El-Khatib et al, 1995). We recently showed that antiestrogens have the potential to affect the Fas pathway in some tumors. This was demonstrated by a significant amplification by antiestrogens of anti-Fas-mediated cytolysis of freshly isolated ovarian carcinoma cells as well as a trend for Fas receptor upregulation in a number of cases after antiestrogen treatment. In a few patients, antiestrogen augmentation of anti-Fas-mediated cytolysis was noticed in spite of unchanged Fas receptor levels (Haeryfar et al, 1999b). It is known that many Fas-expressing tumor cells (e.g. human prostate tumor cell lines PC-3, DU145, LnCAP) are resistant to the cytotoxic anti-Fas antibody (Frost *et al*, 1997). Besides, levels of Fas expression on tumor cell lines do not always correlate with biological responsiveness to engagement of the Fas receptor (Owen-Shaub et al,1994). Whether or not antiestrogens act on downstream elements of the Fas/FasL pathway has yet to be discovered. Tumor necrosis factor-α which also belongs to the same receptor family as Fas, has been shown to be secreted by activated NK cells and to subsequently kill TNF-susceptible targets (Vujanovic et al, 1996). It is also reported that antiestrogens enhance lipopolysaccharide (LPS)-induced TNF secretion by human monocytes (Teodorczyk-Injeyan et al, 1993). So, one might assume some possible

involvement of TNF in antiestrogen induced amplification of NK cytotoxicity. However, this could not be shown to be the reason for enhanced cytotoxicity in our experimental system since first of all, K562 is known to be insensitive to TNF-mediate lysis (Roger et al, 1996), and secondly, apoptosis induced by TNF-α is relatively slow and thus cannot be measured in short term assays (Gardiner and Reen, 1998). Whether or not antiestrogens exhibit any influence on killer cell-mediated cytolysis in long term assays remains to be tested.

III. Antiestrogens, Perforin/Granzyme Pathway and NK Cell-mediated Cytolysis of K562

The constitutive presence of perforin and granzyme B mRNA in $CD3^-$ natural killer cells has been clearly demonstrated by *in situ* hybridization (Clement et al, 1990). In fact, the perforin/granzyme pathway appears to be the predominant pathway in PBMC-derived NK cell-induced cytolysis. The PFN, and granzyme A and B mRNAs are highly expressed in resting NK cells as compared to the FasL mRNA (Mori S, 1997). Both PFN and Grzs are necessary to induce apoptosis in target cells. Our data indicate that NK cell-mediated cytolysis of K562 is almost exclusively due to the Ca^{++}-dependent PFN/Grz pathway. This is demonstrated by total abrogation of cell death when extracellular Ca^{++} is chelated by EGTA/Mg^{++}. Extracellular calcium cations are essential for

exocytosis of granules and insertion and polymerization of PFN on target cell membrane (Berke, 1995). That cytotoxicity in antiestrogen treatment groups was also reduced to the background level in the presence of EGTA suggests that the PFN/Grz pathway is affected by antiestrogens in the case of K562 cytolysis. Treatment of effector cells with the general serine protease inhibitor DCI which is often used as an inhibitor of granzymes in intact cells (Vermijlen et al, 1999) , also significantly but not completely reduced NK-CMC, while cytotoxicity in antiestrogen treatment groups was not decreased to the background level, suggesting that either PFN or enzymes other than granzymes might play a role in antiestrogenic augmentation of NK-CMC. Perforin alone can induce cell death of necrotic nature in target cells and one possibility is that antiestrogens increase the expression of PFN in effector cells. Greenberg and coworkers also showed that preincubation with DCI blocked fragmentin-induced DNA damage in the target, but had no effect on cytolysin (perforin) (Shi et al, 1992).On the other hand, it has been shown recently that the presence of PFN is also required for the induction of apoptosis in the target. GrzB could enter the target cells independently of PFN, possibly through receptor-mediated endocytosis. In the absence of PFN, GrzB shows a cytoplasmic localization. When PFN is added, it presumably enters the cells, through an unidentified mechanism, and GrzB

relocalizes to the nucleus. Cells that show GrzB relocalization undergo apoptotic death (Vermijlen et al, 1999). It is well-known that the cleavage of cytosolic and nuclear substrates by GrzB results in the characteristic apoptotic phenotype. Key among these substrates is a family of cytoplasmic caspases that mediate cell suicide (Trapani et al, 1998). Caspases are a family of cysteine aspartate proteinases whose activation takes place in both Fas/FasL and PFN/Gzm pathways of immune cytolysis (Atkinson et al, 1998). We found that the broad spectrum caspase inhibitor zVAD-fmk was not able to block NK-CMC, but rather unexpectedly increased the cell death regardless of antiestrogenic treatment, suggesting that NK cell-mediated K562 cytolysis and its antiestrogenic augmentation are caspase-independent. Similarly, Vercammen et al considered unexpected their finding that zVAD-fmk rendered Fas transfected L929 fibroblasts more sensitive to Fas-mediated cell death (Vercammen et al, 1998). The caspase-independent nature of killer cell-mediated cytolysis has been previously reported. Trapani et al showed that cell membrane leakage in response to purified PFN and GrzB was independent of caspase activation, although nuclear events such as DNA fragmentation and disintegration were abolished by zVAD-fmk. Interestingly, zVAD-fmk-treated cells exposed to both PFN and GzmB uniformly died when they were re-cultured, whereas cells

exposed to PFN or Gzm alone survived and proliferated as readily as untreated cells (Trapani et al, 1998b). It was also shown in CTLs that cytolysis via the granule exocytosis pathway was completely resistant to caspase inhibitors (Sarin et al, 1998). Andrade and coworkers demonstrated that two of the downstream substrates for the caspase family of proteases during apoptosis, namely DNA-PK_{cs} and NuMA are directly and efficiently cleaved by GzmB both in vitro and in target cells undergoing lymphocyte granule-induced cytotoxicity, confirming the existence of efficient caspase-independent proteolytic pathways during this form of cell death (Andrade et al, 1998). All together, our results suggest an antiestrogenic impact on the PFN/Gzm pathway of NK cell-mediated cytolysis of K562, which is caspase-independent and totally resistant to caspase inhibitors. The molecules or steps which are involved in this pathway and are targeted by antiestrogenic drugs resulting in an enhanced cytolytic phenomenon remain to be identified.

IV. Antiestrogens and LAK Cell-mediated Cytolysis of K562

Lymphokine-activated killer (LAK) cells also use perforin, FasL and TNF as cytotoxic molecules to destroy target cells (Lee et al, 1996). Interleukin-2 is known to augment the cytotoxic capability of both natural killer cells and cytotoxic T lymphocytes. Fas ligand mRNA is minimally expressed in resting

NK cells and is upregulated by IL-2 (Mori et al, 1997). IL-2 upregulates the expression of granzyme B mRNA in NK cells, T lymphocytes with NK-like activity, and CTL clones as well (Fitzpatrick et al, 1996). According to our results, LAK cell-induced cytolysis of K562 also occurred through the perforin/granzyme pathway since it was completely blocked by chelation of extracellular Ca^{++}. Antiestrogen augmentation of LAK cell-mediated cytotoxicity has been previously reported (Baral et al, 1996b and 1996c). We were intrigued by the finding that when human peripheral blood lymphocytes were stimulated *in vitro* with IL-2, antiestrogen treatment downregulated their cytotoxic activity with an overall inhibition when both targets and effector cells were pretreated. One possibility is that the presence of antiestrogens may interfere with IL-2 activation of certain subpopulations of PBLs prior to cytotoxicity assays. This problem could be solved by using purified NK cells instead of PBLs for LAK generation. On the other hand, it is reported that K562 target cells express FasL and are able to fight back by destroying IL-2 activated NK cells. The mechanism involves signalling through integrins and requires *src* family PTK and protease activities. Engagement of other cell surface molecules, such as Fas, may also trigger LAK cell apoptosis under the appropriate conditions (Yamauchi et al, 1996). IL-2 induces Fas expression on NK cells and anti-Fas cytotoxic antibody

can induce apoptosis in IL-2 activated NK cells (Mori et al, 1997). Therefore, it is likely that antiestrogens synergise the IL-2 effect on Fas expression in NK cells, rendering LAK cells more susceptible to K562-induced cell death. Last but not the least, based on our observation, one might hypothesize that antiestrogens interfere with LAK generation by inhibiting IL-2-induced biosynthesis of granzyme B and/or perforin. Little is known about the mechanism(s) by which granzyme B gene expression in cytolytic effector cells is downregulated (Fitzpatrick et al, 1996). Moreover, there is not much understood about the interaction, if any, between hormones/antihormones and perforin/granzyme expression in immune-derived killer cells. It was shown lately that dexamethasone, a glucocorticoid that is widely used as an immunomodulatory and anti-inflammatory agent, inhibits granzyme B mRNA transcription in phytohemagglutinin-activated peripheral blood mononuclear cells. Dexamethasone was able to abrogate the transcriptional activity of the human granzyme B gene promoter by inhibiting the binding of nuclear factors at the AP-1 and Ikaros sites (Wargnier et al, 1998). Whether or not antiestrogens exert a similar impact on granzyme B gene expression has yet to be discovered.

V. Antiestrogens and Anti-Fas-mediated Cytolysis of other Tumor Targets

We also tested the hypothesis that antiestrogens sensitize other tumor targets

with different characteristics in terms of Fas expression and NK/LAK-sensitivity. Of the few targets we examined, Yac-1, P815 and SL2-5 had previously been reported to be sensitized by antiestrogens for killer cell-mediated cytotoxicity (Baral et al, 1995 and 1996). Here, we did not detect any substantial change in target sensitivity to anti-Fas cytotoxic antibodies upon antiestrogenic treatment. However, the involvement of the perforin/granzyme pathway in this connection was not tested and thus cannot be ruled out.

VI. General Conclusion

Antiestrogens are capable of enhancing killer cell-mediated cytotoxic reactions. Previous works have shown that the Fas/Fas ligand pathway of immune cytolysis could be amplified by antiestrogens. K562 erythroleukemia cells are Fas negative and antiestrogenic treatment does not make them susceptible to Fas-mediated killing, yet NK cell-mediated cytolysis of K562 could be significantly augmented by antiestrogens. Our results clearly indicate that the perforin/granzyme pathway of immune cytolysis is influenced in a caspase-independent manner by antiestrogens towards enhanced K562 cytolysis. This could be considered the first report linking antiestrogens to the perforin/granzyme pathway employed by cytotoxic effector cells against tumor targets. The exact steps/elements of the pathway on which antiestrogens act are

unknown at the present time and expected to be discovered.

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