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UNIVERSITY OF ALBERTA

FUNCTIONAL ANALYSIS OF CYTOTOXIC T LYMPHOCYTE-MEDIATED LYSIS

BY

CHERYL D. HELGASON

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitiled **"Functional Analysis of Cytotoxic T Lymphocyte-Mediated Lysis"** submitted by Cheryl D. Helgason in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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ABSTRACT

The mechanism by which cytotoxic T lymphocytes (CTL) lyse their targets has not been elucidated. Exocytosis of cytolytic granules containing perforin and the cytotoxic cell proteinases (CCPs) is the favored model. However, some evidence suggests that alternate mechanisms are also employed. In addition, the role of DNA fragmentation in target cell lysis has been the subject of considerable controversy. The objective of the studies presented in this thesis was to characterize the processes involved in CTL-mediated target cell death.

No evidence for perforin or CCP1-CCP5 expression was detected using the polymerase chain reaction in five hybridomas exhibiting varying levels of cytolytic activity. Two of these hybridomas were chosen for further analysis. It was demonstrated that lysis required protein synthesis and was reduced by inhibitors of poly(ADP-ribosyl)synthetase. Cytotoxicity was not mediated by tumor necrosis factor. However, involvement of a serine proteinase was demonstrated. DNA fragmentation occurred in conjunction with lysis, but did not precede it. These observations suggest a mechanism distinctly different from lysis involving cytolytic granules.

An assay, referred to as the "programming for lysis assay", was developed to examine the contribution of DNA fragmentation to target cell death following minimal exposure to the CTL. In addition, this protocol was used to examine the effects of various agents on lysis in the absence of CTL.

The endonuclease inhibitor aurintricarboxylic acid (ATA) effectively inhibited both chromium release and DNA fragmentation in the target cell. There was no evidence for nonspecific effects on either the CTL or the target. Furthermore, inhibition of DNA fragmentation correlated with an increase in target cell viability, suggesting that DNA damage contributes to target cell death.

Results with three proteinase inhibitors suggested a dual role for the CCPs. In the early stages of target cell death they participate in DNA fragmentation. They also appear to have a continuing role in the processes which lead to loss of integrity of target cell membranes.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

ANA	4-amino-1,8-napthalimide
ATA	Aurintricarboxylic acid
ATEE	N-acetyl-L-tyrosine ethyl ester
BAEE	N-benzoyl-L-arginine ethyl ester
BLT	Nα-benzyloxycarbonyl-L-lysine thiobenzyl ester
BSA	Bovine serum albumin
CCP	Cytotoxic cell proteinase
CD	Cluster of differentiation
cDNA	Complementary DNA
ConA	Concanavalin A
Cr	Chromium
CsA	Cyclosporin A
CTL	Cytotoxic T lymphocyte
DCI	3,4-dichloroisocoumarin
DFP	Diisopropyl fluorophosphate
DMSO	Dimethyl sulfoxide
DNA	Deoxynucleic acid
dNTP	Deoxynucleoside triphosphate
DTNB	5,5'-dithiobis(2-nitrobenzoic acid)
DTT	Dithiothreitol
E:T	Effector : Target ratio
EDTA	Ethylenediamine tetraacetic acid
EGTA	Ethyleneglycol-bis-(ß-aminoethylether) tetraacetic acid
FF	Formamide/formaldehyde
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid

HF	Hanukah factor
ICAM	Intercellular adhesion molecule
IL	Interleukin
KCIL	Killer cell independent lysis
kDa	Kilodalton
LAK	Lymphokine activated killer cell
LFA	Lymphocyte function-associated protein
LGL	Large granular lymphocyte
LT	Lymphotoxin; TNF-β
MHC	Major histocompatibility complex
MLC	Mixed lymphocyte culture
MLR	Mixed lymphocyte reaction
MOPS	3-(N-morpholine)propanesulfonic acid
mRNA	Messenger ribonucleic acid
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
NK	Natural killer cell
NKCF	Natural killer cytotoxic factor
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PEL	Peritoneal exudate lymphocyte
SD	One standard deviation
SDS	Sodium dodecyl sulfate
TcR	T cell antigen receptor
TNF	Tumor necrosis factor
UV	Ultraviolet

CHAPTER 1: INTRODUCTION

The role of the immune system is to provide protection against invading organisms, as well as to prevent tumor growth. There are two branches of the immune system. Humoral immunity is mediated by B lymphocytes which produce antibodies to protect the body against invading bacteria and viruses. Cellular immunity, mediated primarily by T lymphocytes, requires intimate contact between the effector cell of the immune system and the infected or cancerous cell. This branch of the immune system protects the host from viral infections, as well as from oncogenic cells and foreign tissues.

The cellular immune response requires a coordinated effort by numerous cells. Helper T lymphocytes produce a variety of soluble growth factors called lymphokines which modulate the activity of T lymphocytes as well as other cells of the immune system. For example, interleukin 2 (IL2) is required for the maturation and/or proliferation of cytotoxic T lymphocytes. In contrast to helper T cells, suppressor T lymphocytes down-regulate the immune response once infection is under control. Helper and suppressor, as well as cytotoxic, T lymphocytes recognize and respond to portions of foreign proteins, broken down into peptides called antigens, presented on the surface of infected cells. Virtually every cell in the body has the capacity to participate in the cellular immune response by presenting antigen to T lymphocytes.

An overview of the field of immunology, as it relates to the cellular immune response, is provided in this introduction. Emphasis is placed on those subjects required to understand the work presented in this thesis.

EFFECTORS OF THE CELLULAR IMMUNE RESPONSE

Several different types of cells are responsible for the effector activity of cell-mediated immunity. Cytotoxic T lymphocytes, peritoneal exudate lymphocytes, natural killer cells, and lymphokine activated killers are involved in the destruction of virus-infected and tumor

cells in the body. This section describes in detail the effectors capable of destroying infected or cancerous cells.

Cytotoxic T lymphocytes (CTL)

CTL are one of the most specific effectors of the cell-mediated immune response. These cells originate in the bone marrow and continue the maturation process in the thymus (thus the name "T" lymphocyte). It is here that CTL learn to differentiate between self and foreign antigens (Grey *et al.*, 1989). In this pathway, known as selection, T cells which recognize self antigens are removed through activation of a pathway leading to programmed cell death (apoptosis). Those T cells which recognize foreign antigens are selected for further maturation. Once these precursor cells have been through this process of selection, they exit the thymus and await the final stages of maturation and activation in secondary lymphoid tissues.

CTL express a number of characteristic surface antigens which distinguish them from other cell types. The most significant feature is an immunoglobulin-like receptor known as the T cell antigen receptor (TcR) consisting of one acidic alpha chain (39-46 kDa) and one basic beta chain (40-44 kDa) (Allison and Lanier, 1987). The TcR is involved in the specific recognition and binding of antigen presented in association with class I major histocompatibility complex molecules (MHCI). Antigen can only be recognized by the TcR when it is present on the cell surface in association with MHC molecules. This is the concept of "MHC restriction" exhibited by all classes of T lymphocytes. MHCI, as indicated earlier, is expressed by most cells in the body.

Associated with the TcR is an invariant multimeric complex called CD3 composed of five different proteins (Weiss *et al.*, 1986). The core consists of the gamma, delta, and epsilon molecules. The zeta chain associates with the TcR/CD3 complex as either a homodimer or as part of a heterodimer with the eta chain. In contrast to the alpha and beta chains of the TcR, the CD3 proteins have long cytoplasmic domains which permit association with various signal transduction pathways within the CTL.

In addition to the TcR, CTL have cell surface proteins known as lymphocyte function-associated antigens (LFA). These molecules play a role in the primary adhesions formed between CTL and target cells. Clusters of differentiation (CD) (Springer *et al.*, 1987) are also surface molecules which distinguish the various cells of the immune system from one another. For example, helper T lymphocytes usually express CD4, while CTL express CD8. CD4 molecules associate with MHC class II on target cells and CD8 molecules associate with MHC class I. This association adds a further differentiation between helper and cytotoxic T cells. The roles of the CDs vary from one protein to the next. In general, they are involved in both adhesion and signal transduction.

CTL maintained *in vitro* are small cells containing a large number of electron dense granules within their cytoplasm, the significance of which will be discussed later. Mature CTL are TcR^+, $CD8^+$, $CD2^+$ cells which express LFA-1. However, some $CD4^+$ cells, traditionally considered helper cells, have the capacity to function as cytolytic cells (Ju, 1991; Lancki *et al.*, 1991; Smyth *et al.*, 1992). Although they share some properties with $CD8^+$ cells, they differ significantly in certain aspects and will not be discussed in any detail.

A CTL becomes activated to proliferate or lyse targets, depending upon its state of maturation, following association of the TcR with foreign antigens. This effect can also be mimicked by stimulation of the TcR with mitogenic lectins such as Concanavalin A (ConA) or antibodies directed against the CD3 complex (Mueller *et al.*, 1989). As mentioned above, CTL do not recognize free antigen. Instead, the antigen must be presented as a peptide with MHCI molecules on the surface of the target cell (Grey *et al.*, 1989). This association between TcR and antigen triggers a number of biochemical events which will be discussed later as they relate to their role in CTL-mediated lysis.

Peritoneal exudate lymphocytes (PEL)

PEL are small to medium-sized nondividing lymphocytes found at the rejection site of tumor allografts (Berke, 1987). These *in vivo* CTL are capable of potent, specific cytocidal activity. They arise from the same precursor cells as CTL and express the characteristic CTL markers including CD8, CD3, and the TcR. Unlike CTL cultured *in vitro*, PEL are agranular and do not express detectable hemolytic (perforin) or benzyloxycarbonyl-L-lysine thiobenzyl ester (BLT) esterase (granzyme A) activity (Berke and Rosen, 1987; Dennert, 1987). Detection of MRNA for these proteins in primary PEL has made this last point highly controversial (Brunet *et al.*, 1987; Nagler-Anderson *et al.*, 1989; Ebnet *et al.*, 1991).

Certain characteristics of lysis mediated by PEL are indistinguishable from those induced by granule-containing CTL (Allbritton *et al.*, 1988a). Culture of primary or secondary PEL cells in vitro with IL2 results in blast formation with the rapid accumulation of cytoplasmic granules (Berke, 1987; Berke and Rosen, 1988). This acquisition correlates with the appearance of both perforin and BLT esterase activity. These results suggest that mature CTL possess more than one mechanism of lysis. The predominant lytic inducer may vary depending on the maturation state of the CTL or on the target with which it is confronted.

Natural killer cells (NK)

NK cells function as the first line of defense against viral infection. Infected cells produce interferons which stimulate a consequent recruitment of NK cells (Burns *et al.*, 1985). Although their precise origin has not been determined, they are non-B, non-T lymphocytes which develop from the same lymphoid precursor stem cells which give rise to CTL (Janeway, 1991). Unlike CTL, NK cells do not express the TcR. Thus, they are not MHC-restricted and exhibit a broad range of target specificities.

In the absence of a TcR, it unclear how NK cells recognize virus-infected targets. They appear to specifically contact those cells

expressing low levels of MHCI suggesting that MHC molecules normally mask the target structures recognized by NK cells (Roder, 1991). This recognition may be mediated, in part, by a 1409 amino acid receptor encoded by an NK-specific, low abundance, 7 kilobase MRNA (Roder, 1991).

Morphologically, NK are large granular lymphocytes (LGL) with a $CD3^-$, $CD8^-$, $CD16^+$, $CD56^+$ surface phenotype (Trinchieri, 1989). In addition, they express the IL2 receptor and a marker for prothymocytes known as asialo-GM1 (Kaplan, 1986). Fc receptors for immunoglobulin G enable NK to lyse targets previously coated with antibody in a powerful lytic mechanism described as antibody-dependent cellular cytotoxicity (Young, 1989).

Lymphokine activated killer cells (LAK)

Some LAK cells arise from specific subsets, $NK1.1^+/LGL\text{-}1^-$, of NK cells following in vitro culture in high levels of the growth factor IL2 (Mason *et al.*, 1990). In addition, long-term culture with anti-CD3 antibody can result in the generation of $CD3^+$ LAK from both $CD4^+$ and $CD8^+$ CTL (Smyth *et al.*, 1991). Induction of LAK activity is inhibited by transforming growth factor-beta (Grimm *et al.*, 1988).

Morphologically and phenotypically, LAK cells resemble the NK or CTL from which they are derived. They are large lymphocytes with an abundance of cytoplasmic granules. LAK cells, like NK, are not MHC-restricted but they have the capacity to lyse a wider variety of target cells (Hiserodt and Chambers, 1988).

CYTOLYTIC MOLECULES

The molecules utilized to induce target cell lysis are as diverse as the effectors which employ them. They can be loosely categorized into two groups. The first, perforin and the cytotoxic cell proteinases (CCPs), are located within the granules of CTL, NK, and LAK. The second group of mediators are soluble factors such as tumor necrosis factor (TNFα), lymphotoxin (LT), leukalexin, and NK cytolytic factors (NKCFs).

Cell surface TNFα and LT are also included with this group. The properties of these lytic molecules are outlined below.

Perforin

Perforin was one of the first proteins isolated from cytolytic granules, although its presence was postulated before its identification. Dourmashkin *et al.* (1980) detected discrete pores in the membranes of antibody-covered erythrocyte ghosts following exposure to CTL. These initial observations were later confirmed and extended using both NK and CTL against normal targets (Dennert and Podack, 1983; Podack and Dennert, 1983).

Perforin (also known as cytolysin or pore-forming protein) was subsequently isolated from the granules of both CTL and NK cells (Masson and Tschopp, 1985; Podack *et al.*, 1985; Liu *et al.*, 1986). Monomeric perforin has a molecular mass of 70 kilodaltons (kDa) under reducing conditions, 62 kDa under nonreducing conditions. Purified perforin forms the same type of channels as those observed using isolated granules (Henkart *et al.*, 1984; Podack and Konigsberg, 1984). In the presence of calcium, perforin oligomerizes into multimeric tubular structures which associate with phosphorylcholine moieties on the target cell membrane (Tschopp and Nabholz, 1990). Insertion of the oligomer occurs so that the hydrophobic portions of the molecule face outwards toward the cell membrane while the hydrophilic portions line the core of the tubule (Stanley and Luzio, 1988). Each channel consists of 12-18 perforin protomers joined together to form a tubule 16 nm high and 5-20 nm in diameter (Tschopp and Nabholz, 1990).

The perforin cDNAs, isolated from both human and mouse, share 69% identity (Shinkai *et al.*, 1988; Lichtenheld *et al.*, 1989; Tschopp and Nabholz, 1990). The gene encoding human perforin has also been isolated, characterized, and localized to chromosome 17 (Lichtenheld and Podack, 1989; Shinkai *et al.*, 1989). These sequences predict a protein 534 amino acids in length with a 20 amino acid leader sequence and three potential N-glycosylation sites. Perforin has some homology with various components of the complement cascade. This group of proteins

cooperates to form pores within target cell membranes during the humoral immune response. The greatest similarity is observed in the two regions of perforin which correspond to the membrane-spanning domain and the cysteine-rich region of the pore-forming C9 protein of complement (Podack *et al.*, 1991).

Perforin is expressed by most $CD8^+$ and some $CD4^+$ killer cells, as well as by NK and LAK cells. *In vitro* there is a correlation between perforin expression and acquisition of cytolytic activity (Lichtenheld *et al.*, 1988; Kawasaki *et al.*, 1990). *In vivo* perforin is found in situations where specific CTL-mediated cytotoxicity would be expected. For example, *in situ* hybridization reveals perforin mRNA during an immune response against viral infection, in autoimmune disorders, at the site of transplant rejection, and at tumor locations (Mueller *et al.*, 1988b; Mueller *et al.*, 1989; Podack *et al.*, 1991).

Two further pieces of evidence, in addition to the expression studies, suggest a role for perforin during cytolysis. First, antisense oligonucleotides which prevent translation of perforin in CTL also partially inhibit target cell chromium release (Acha-Orbea *et al.*, 1990). Furthermore, transfection of perforin expression vectors into rat basophilic leukemia cells confers the ability to lyse red blood cells and antibody-modified tumor cells (Shiver and Henkart, 1991). Perforin may also function to permit the entry of other granular proteins, such as the serine proteinases, into the target cell (Podack *et al.*, 1988).

Cytotoxic cell proteinases (CCPs)

The involvement of serine proteinases in CTL and NK mediated lysis was suggested by the inhibition of killing using a variety of proteinase inhibitors (Chang and Eisen, 1980; Redelman and Hudig, 1980; Hudig *et al.*, 1981; Pasternack *et al.*, 1983). The initial demonstration of proteinase activity associated with CTL came from the studies of Pasternack and Eisen (1985) who found high levels of a CTL-specific BLT esterase activity.

Two general approaches lead to the identification of several CTL-specific serine proteinases. The first involved isolation and characterization of proteins present within the granules of CTL and NK. The second was a molecular biology approach involving differential screening of CTL cDNA libraries with probes from both CTL and noncytolytic cells. The combined results of these investigations culminated in the identification of seven serine proteinases in the mouse and four in humans. Table 1.1 provides a summary of the murine serine proteinase family.

Granzyme A (HF) was the first serine proteinase isolated and characterized from murine CTL (Gershenfeld and Weissman, 1986; Masson *et al.*, 1986). The protein is a dimer with a molecular mass of 60 kDa, 35 kDa in the reduced state. HF is the proteinase responsible for the BLT-esterase activity initially detected in CTL (Pasternack and Eisen, 1985; Kramer *et al.*, 1986). It differs significantly from the remainder of the granule serine proteinases in sequence, gene structure, chromosomal location, and protein properties. Therefore, it was not given a CCP designation (Bleackley, 1988b).

HF is the only CTL serine proteinase for which natural substrates have been identified. It binds to and cleaves nucleolin, a protein involved in transport between cytoplasm and nucleus, as well as casein, fibrin, and extracellular matrix proteins (Masson and Tschopp, 1988; Pasternack *et al.*, 1991). In addition, HF also mediates DNA fragmentation in target cells permeabilized with detergent or sublethal doses of perforin (Munger *et al.*, 1988; Hayes *et al.*, 1989; Shi *et al.*, 1992a). However, the kinetics of induction are slow compared to the time required to observe DNA damage caused by intact CTL. Co-transfection of the rat basophilic leukemia cell line with both perforin and HF results in target cell lysis, with DNA fragmentation observed in as little as four hours (Shiver *et al.*, 1992). Therefore, it is possible that HF is involved in the DNA damage observed during CTL-mediated lysis.

Table 1.1: Summary of murine cytotoxic cell proteinases.

Proteinase	Synonym	Mass (kDa)	Reference
HF	Granzyme A	35(R)* 60(NR)	Pasternack & Eisen, 1985; Masson *et al.*, 1986a; 1986b; Gershenfeld & Weissman, 1986
	SE1		Young *et al.*, 1986
	CTLA-3		Brunet *et al.*, 1986
	TSP-1		Simon *et al.*, 1986
CCP1	Granzyme B	29	Lobe *et al.*,1986a; 1986b; 1988; Masson and Tschopp, 1987
	SE2		Young *et al.*, 1986
	CTLA-1		Brunet *et al.*, 1986
CCP2	Granzyme C	27	Lobe *et al.*,1986a; 1986b; 1988; Jenne *et al.*, 1988a
CCP3	Granzyme E	35-50	Bleackley *et al.*, 1988a; 1988b; Jenne *et al.*, 1988b; Prendergast *et al.*, 1991
	MCSP-2		Kwon *et al.*, 1988
CCP4	Granzyme F	35-45	Bleackley *et al.*, 1988a; 1988b; Jenne *et al.*, 1988b, 1991; Prendergast *et al.*, 1991
	MCSP-3		Kwon *et al.*, 1988
CCP5	Granzyme D	35-40	Bleackley *et al.*, 1988a; 1988b; Jenne *et al.*, 1988b; Prendergast *et al.*, 1991
CCP6	Granzyme G	33	Masson and Tschopp, 1987; Jenne *et al.*, 1989

* R = reduced; NR = nonreduced

References listed are for proteins, cDNA clones, and genomic clones using the CCP designation of Bleackley *et al.* (1988b) and the granzyme designation of Masson *et al.* (1986a, 1987).

Human HF (HuHF, CSPA, HuTSP) is similar to murine HF in both molecular properties and substrate specificity. It has been characterized at the protein, cDNA and genomic levels (Fruth *et al.*, 1987; Ferguson *et al.*, 1988; Gershenfeld *et al.*, 1988b; Krahenbuhl *et al.*, 1988). HuHF is a dimer with a molecular mass of 50-51 kDa (nonreduced). The monomer has a mass of 27-31 kDa.

The remainder of the CTL-specific serine proteinases have a CCP designation due to their high degree of amino acid identity, localization to the same chromosomal position, and the similarity of their features at the protein level (Masson and Tschopp, 1987; Bleackley *et al.*, 1988b; Tschopp and Jongeneel, 1988). All contain the His, Asp, and Ser residues which make up the characteristic catalytic triad of serine proteinases. The Ser residue is found as part of the conserved sequence Asp-Ser-Gly-Gly. In addition, each is translated as a prepropeptide with a 20 amino acid leader sequence. This is removed to yield a zymogen form of the proteinase with the acidic dipeptide Gly-Glu or Glu-Glu present at the N-terminus. The final cleavage removes this dipeptide to yield an active proteinase with the conserved N-terminal sequences Ile-Ile-Gly-Gly.

These basic proteinases, like perforin, associate with chondroitin sulfate A proteoglycans in the acidic cytolytic granules of CTL and NK (Masson *et al.*, 1990). The proteoglycans are released, possibly as proteoglycan-proteinase complexes, during the process of granule exocytosis associated with target cell lysis (Schmidt *et al.*, 1985). The neutral pH environment of the synapse between effector and target causes dissociation of the complex to release active proteinases or perforin (Tschopp and Jongeneel, 1988).

CCP1 and CCP2 were originally identified as cDNA clones (Lobe *et al.*, 1986a; 1986b; Jenne *et al.*, 1988a). Both have been extensively characterized at the genetic level and elements involved in the regulation of their expression have been partially identified (Lobe *et al.*, 1989; Fregeau and Bleackley, 1991). Although the natural substrates have not been identified for either of these proteins, synthetic substrates have been characterized for CCP1. As predicted by

molecular modelling (Murphy *et al.*, 1988) this enzyme cleaves its substrate after an Asp residue and is therefore called an aspase (Odake *et al.*, 1991). The rat homolog of CCP1, designated Fragmentin 2, induces DNA fragmentation in target cells exposed to sublethal doses of perforin (Shi *et al.*, 1992a; 1992b). Significant levels of DNA damage are induced within 2 hours of initial contact suggesting a role in the lysis observed during *in vitro* cytotoxicity assays.

Human CTL also express CCP1 (HuCCP1) (Schmid and Weissman, 1987; Caputo *et al.*, 1988; 1990; Trapani *et al.*, 1988; Krahenbuhl *et al.*, 1988; Poe *et al.*, 1991). Expression of HuCCP1, in a manner similar to murine CCP1, is induced during the generation of a cytolytic response. A third human CTL-specific serine proteinase is human CCPX (HuCCPX), also designated Granzyme H (Meier *et al.*, 1990; Haddad *et al.*, 1991). Although the cDNA of HuCCPX resembles CCP1 at its 5′ end, the 3′ end differs from the other characterized proteinases. A mouse homolog of HuCCPX has yet to be found. The genes for both HuCCP1 and HuCCPX are localized on chromosome 14 (Klein *et al.*, 1989; Lin *et al.*, 1990). The relationship of the fourth human proteinase, designated Granzyme 3, to the others has not been determined (Hameed *et al.*, 1988). This is due, in part, to the fact that only the N-terminal amino acid sequence has been determined. However, its trypsin-like activity suggests that it may be the homolog of mouse CCP5 and rat fragmentin 3 (Shi *et al.*, 1992a).

A novel serine proteinase, designated RNKP-1, was isolated from the granules of a rat NK cell line (Zunino *et al.*, 1990). RNKP-1 shares 85% identity with mouse CCP1 and also has aspase activity. It was recently demonstrated that this proteinase has a potent cytostatic (growth inhibitory) activity (Sayers *et al.*, 1992). Further characterization of this protein is required.

The remainder of the mouse proteinases share extensive homology with each other, as well as with CCP1 and CCP2 (Bleackley *et al.*, 1988b; Jenne *et al.*, 1988b; Kwon *et al.*, 1988). For example, cDNA sequence comparisons reveal 64-95% identity. Analysis of protein sequences suggests 56-90% identity. In contrast, the extent of similarity between

HF and the CCP family never exceeds 48%, even at the cDNA level. CCP3, CCP4, and CCP5 are glycosylated to varying degrees, thus explaining the range of molecular masses recorded for these proteins (Table 1.1). The natural substrates have not yet been found for any of these proteinases. However, studies with synthetic substrates suggest that CCP5, like HF, also has trypsin-like activity (Jenne and Tschopp, 1988; Odake *et al.*, 1991). In addition, purified preparations of the rat CCP5 homolog (Fragmentin 3) have the ability to induce DNA fragmentation with a delayed onset in target cells exposed to low levels of purified perforin (Shi *et al.*, 1992a).

The genes for the CCPs have also been isolated and characterized (Lobe *et al.*, 1988; Jenne *et al.*, 1991; Prendergast *et al.*, 1991; 1992a). Each consists of 5 exons interrupted by 4 introns of similar sizes and locations. Chromosomal positions have been assigned to five of the CCPs. All are clustered on chromosome 14, closely linked to the genes for the alpha and beta chains of the TcR (Brunet *et al.*, 1986; Crosby *et al.*, 1990; Hanson *et al.*, 1990). In contrast, the gene encoding HF is located on chromosome 5.

Patterns of serine proteinase mRNA expression have been extensively examined. Granzyme A is detected in B cells, lung cells, intestinal cells, and in noncytolytic cells of lymphoid origin, as well as in cytolytic cells (Lobe *et al.*, 1986b; Brunet *et al.*, 1987; Velotti *et al.*, 1989; Garcia-Sanz *et al.*, 1990). CCP1 and CCP2 are found almost exclusively in activated CTL, NK and LAK cells (Brunet *et al.*, 1987; Bleackley, 1988; Manyak *et al.*, 1989; Velotti *et al.*, 1992).

In general, maximal levels of CCP1 and CCP2 mRNA are observed prior to the peak of cytolytic activity in a mixed lymphocyte reaction (MLR) or following Concanavalin A (ConA) stimulation of mouse spleen cells (Lobe, 1986b; Bleackley *et al.*, 1988a). However, not all members of the proteinase family are induced in response to a particular stimulus (Garcia-Sanz *et al.*, 1990; Ebnet *et al.*, 1991; Prendergast *et al.*, 1992b). For example, following stimulation of mouse splenocytes with anti-CD3 monoclonal antibody (∝CD3 mAb) expression of CCP1 is observed prior to the peak of cytolytic activity. CCP2 mRNA is not

detected until activity has significantly declined. CCP3, CCP4, and CCP5 are not expressed in the six days following mAb stimulation. These results, along with the distinct substrate specificities of each proteinase, suggest that in spite of the extensive homologies amongst the family members, each must have a unique function.

Serine proteinase expression has also been detected *in vivo*. HF and CCP1 were found by *in situ* hybridization in lymphocytes obtained from the sites of primary allograft rejections and in lymphocytes induced by viral infection (Mueller *et al.*, 1988a; 1988b; 1989; Welsh *et al.*, 1990).

Tumor necrosis factors (TNF, LT)

Two closely related molecules, TNFα (TNF) and lymphotoxin (LT; TNF-β) are expressed by a variety of cell types, including CTL (Vassali, 1992). LT is a 25 kDa glycoprotein with a core protein mass of approximately 18.5 kDa (Tschopp and Jongeneel, 1988). TNFα is a 17.3 kDa protein (Pennica *et al.*, 1985). These two molecules share extensive homology at the amino acid level. In addition they both bind to the same receptors, TNF-R1 (55 kDa) and TNF-R2 (75 kDa).

The characteristics of cell death induced by the TNFs differ from those caused by CTL. For example, TNFα mediated lysis involves ADP-ribosylation (Agrawal *et al.*, 1988) and the production of toxic superoxide radicals within the target cell (Golstein *et al.*, 1991). The earliest detectable effects on susceptible targets involve disruption of the microfilament lattice, contraction of the cell volume, and inhibition of mitochondrial respiratory function. Tumors *in vivo* and tumor cell lines *in vitro* are sensitive to both the cytostatic (growth-preventing) and cytotoxic (lytic) effects of the TNFs. Most normal cells are resistant.

Lysis is further characterized by DNA fragmentation and involvement of a serine proteinase during the later stages of cell death (Suffys *et al.*, 1988). Treatment of targets with either actinomycin D and/or cycloheximide prior to TNF exposure enhances its lytic activity.

This suggests that proteins are synthesized to protect the cell against the toxic effects of TNF (Golstein *et al.*, 1991; Vassali, 1992).

Both TNF∝ and LT are released as soluble molecules into the extracellular space. Target cell death is not observed until 18-24 hours following exposure. These observations argue against a role for either protein in CTL-mediated lysis. However, an active, membrane-associated 26 kDa form of TNF has been isolated and characterized (Kriegler *et al.*, 1988; Kinkhabwala *et al.*, 1990; Perez *et al.*, 1990). This molecule appears to be a precursor of the soluble protein. Membrane-bound TNF may be involved in contact-dependent CTL-mediated lysis (Birkland *et al.*, 1992), but further investigation is required to assess this possibility.

Leukalexin

Leukalexin is a 50-60 kDa protein immunologically related to the TNFs (Liu *et al.*, 1987). Its activities are partially blocked by antibodies against both TNF∝ and LT. Leukalexin is similar to TNF in other properties as well. It is also a secreted protein which induces DNA fragmentation, detected at 18-24 hours, in a number of different target cells. Various forms of this molecule are found in different cells including CTL, NK, helper T cells, and mast cells (Young, 1989). Unlike perforin, the activity of leukalexin is stable in the presence of divalent cations such as magnesium and calcium. The role of leukalexin in target cell lysis has not been determined.

Natural killer cytotoxic factors (NKCFs)

NKCFs are a family of proteins isolated from the granules of NK cells (Wright and Bonavida, 1982; 1987). They range in size from 15-40 kDa (Bonavida, 1988). However, an anti-NKCF antibody which blocks lysis precipitates a protein of 12-14 kDa under reducing conditions (Ortaldo *et al.*, 1987). This suggests that the family may contain other proteins, or that family members are modified *in vivo*. Fractionation studies reveal two discrete NKCF activities. An acidic fraction has an optimal pH of 5-6, while the neutral pH activity is maximal at pH 7-8.

Antibodies against TNF∝ block a portion of the activity of the acidic form.

Unlike the other soluble mediators, NKCFs display specificity toward their targets by binding to specific sites on the membrane. However, they appear to function in a similar manner. That is, they act as soluble mediators and induce DNA fragmentation with a long latency, approximately 18-40 hours, in their targets (Hiserodt and Chambers, 1988).

M-CTX

M-CTX is a membrane-associated cytotoxic factor recently identified by Felgar and Hiserodt (1992). The activity was found in the perforin-depleted, postnuclear homogenates of LAK cells. This fraction induced chromium release from YAC-1 and P815 targets within 1-4 hours. M-CTX associates with the plasma membrane and is inactivated by both trypsin treatment and heating. Its activity is not blocked by either proteinase inhibitors or antibodies against TNF∝ or LT. In addition, it does not require calcium or magnesium. This factor also induces DNA fragmentation in target cells. Further characterization of this activity, and its expression, are required.

LYTIC MECHANISMS

Numerous investigators have examined the process of target cell lysis induced by CTL and NK. Cytolytic granules were implicated as the major participants for two reasons. First, most of the studies utilized CTL cultured with IL2 *in vitro* - conditions which promote granule acquisition. Secondly, certain aspects of the lytic mechanism were demonstrated using isolated granules from cultured killer cells. These studies resulted in the formulation of one favored and several alternate mechanisms of lysis discussed in detail below.

Stages of the lytic cycle

Regardless of the mechanism of lysis proposed, a number of discrete steps are involved (Martz, 1975; Hiserodt *et al.*, 1982a). These include conjugation between effector and target, delivery of the lethal hit (programming for lysis), and dissociation of the effector with subsequent target cell death during the killer cell independent lysis (KCIL) step. Variations amongst the proposed lytic mechanisms are confined primarily to the second phase, delivery of the lethal hit.

The initial association between a CTL and its target is a magnesium-dependent event mediated by the interaction of LFA-1 and CD2 on the T cell with ICAM-1 and LFA-3 on the target cell (Springer *et al.*, 1987; Podack *et al.*, 1988). These antigen-independent adhesion events are followed by a specific TcR-mediated recognition of antigen presented with MHCI on the target cell. Without this critical interaction, the initial adhesion falls apart and the CTL contacts another potential target cell. Finally, CD8 strengthens the interaction by associating directly with the MHCI molecule. These binding events initiate the signals required to induce proliferation and maturation of precursor T cells, in the presence of appropriate growth factors, or to induce delivery of the lethal hit by mature CTL (Weiss *et al.*, 1986; Mueller *et al.*, 1989).

Several intracellular events are triggered by the intimate contact between CTL and target. Antigen binding to the TcR activates phospholipase C which hydrolyses phosphatidyl 4,5-bisphosphate into diacylglycerol and inositol 1,4,5-trisphosphate (Gardner, 1989). Subsequently, diacylglycerol stimulates protein kinase C resulting in the phosphorylation of numerous intracellular proteins. At the same time, inositol trisphosphate induces the release of calcium from intracellular stores, accompanied by an influx of calcium from the extracellular environment. The net result is an increase in intracellular calcium.

The role of the CD3 complex which is associated with the TcR has not been clearly elucidated. It is likely that the TcR is responsible for antigen recognition and binding, while the CD3 complex transduces

the intracellular signals initiated by association with antigen (Allison and Lanier, 1987). All chains of the CD3 complex become phosphorylated following activation of the CTL. This modification may be necessary for the generation of appropriate signals. The CD8 molecule, associated with a lymphocyte cell kinase ($p56^{lck}$), and CD2 may also play accessory roles in facilitating signal transduction (Hunig *et al.*, 1987; Bierer *et al.*, 1989). The net result of these intracellular changes is dependent upon the maturation state of the cell.

This point is best illustrated by considering the result of antigen stimulation of immature thymocytes, precursor CTL, and mature CTL. Encounter of antigen by immature thymocytes can have two outcomes. If the antigen is a self-antigen signals are generated within the thymocyte which activate the suicide program. This results in removal of potentially self-reactive CTL from the body. Foreign antigen generates signals which prepare the thymocyte for further maturation in the periphery. When a precursor CTL engages an antigen, accessory signals provided by a cell-surface protein on the antigen-presenting cell are required. These signals activate pathways within the CTL resulting in the proliferation of the CTL (in the presence of appropriate growth factors) and the acquisition of cytolytic effector molecules. The result of antigen stimulation of a mature CTL is descibed below.

Antigen binding by a mature CTL results in a number of morphological changes. Although the signals responsible for these changes have not been determined, a role for both the calcium fluxes and protein kinase C activation are proposed (Gray *et al.*, 1987; Engelhard *et al.*, 1988; Haverstick *et al.*, 1991). Shortly after contact with the target cell, there is a reorganization within the cytoplasm of the CTL. The microtubule organizing center (MTOC) and Golgi apparatus reorient toward the point of target cell contact, while the nucleus moves further away (Kupfer and Dennert, 1984; Kupfer *et al.*, 1985). High resolution Nomarski optics cinematography was utilized to visualize the effect of conjugation on the CTL (Yanelli *et al.*, 1986). These studies revealed

a movement of the cytolytic granules toward the point of contact with the target cell.

The second stage of the lytic cycle, delivery of the lethal hit, is the most controversial. Exocytosis of the lytic granules and/or their components is the favored model (Henkart, 1985). However, lysis may occur in the absence of granule release (Ostergaard *et al.*, 1987; Trenn *et al.*, 1987; Young *et al.*, 1987). One suggestion is that changes in calcium levels within the target cell are sufficient to induce death (Tirosh and Berke, 1985). Secreted ATP has also been implicated as a potential lytic mediator (Filippini *et al.*, 1990a; Zanovello *et al.*, 1990). These proposals will be discussed in greater detail in the following sections.

Programming of the target cell for lysis is a calcium-dependent, temperature-sensitive event (Martz, 1975; Golstein and Smith, 1976). This stage of the cycle is inhibited by a number of agents which will be discussed in a following section (Gately *et al.*, 1980; Hiserodt *et al.*, 1982b). Delivery of the lethal hit is estimated to be complete within 10-30 minutes following conjugation between the effector and target cells.

KCIL, the final phase of the lytic cycle, requires neither calcium nor magnesium (Golstein and Smith, 1976; Gately and Martz, 1977). Although the mechanism by which the effector cell dissociates from the target is unknown, serine proteinases may mediate this process (Tschopp and Nabholz, 1990). The CTL, once dissociated, is free to initiate another lytic cycle with a new target. Meanwhile, target cell death occurs over a period of several hours. Few inhibitors have been shown to block the KCIL phase.

Osmotic lysis versus internal disintegration (apoptosis)

The mode of target cell death has also been the center of controversy. Two separate models have been proposed to explain the features of killer cell induced lysis.

Perforin alone induces necrotic death in target cells (Podack and Konigsberg, 1984; Masson and Tschopp, 1985). Characteristics of

necrosis include dilation of the endoplasmic reticulum, an increase in mitochondrial volume, cell swelling, and flocculation of the nuclear chromatin (Wyllie *et al.*, 1980). The first model, the theory of colloid osmotic lysis, was proposed to explain target cell death following the membrane damage induced by perforin-mediated pore formation (Dennert and Podack, 1983; Podack and Dennert, 1983; Henkart, 1985). These pores permit small ions to escape from the intracellular environment. This release is counteracted by an influx of water molecules, resulting in target cell lysis with the characteristics of necrosis.

Observations of CTL-induced death reveal morphological features incompatible with osmotic rupture. Sanderson (1976) first observed that contact with CTL induced violent blebbing of the target cell membrane. Shortly after programming for lysis (i.e., delivery of the lethal hit), the target cell DNA fragments into 150-180 base pair pieces (Russell *et al.*, 1980; Russell and Dobos, 1980). Russell (1983) proposed the second model known as the internal disintegration model to explain CTL-mediated lysis. According to this theory, the CTL induces a series of reactions within the target which culminate in target cell suicide resulting from self-inflicted DNA damage. This process is analogous to the preprogrammed cell death (often called apoptosis) observed during selection and maturation of T cells in the thymus (Shi *et al.*, 1989; Smith *et al.*, 1989; McConkey and Orrenius, 1991).

Apoptosis is characterized by cell surface blebbing, condensation of the chromatin around the periphery of the nucleus, and fragmentation of the DNA into an oligonucleosomal ladder (Wyllie *et al.*, 1980). Although the nuclease responsible for DNA degradation has not been identified, a candidate was isolated from the nuclei of apoptotic thymocytes (Caron-Leslie *et al.*, 1991; Gaido and Cidlowski, 1991). This protein has a calcium and magnesium dependent activity and an apparent molecular mass of 25 kDa. A second candidate, with a molecular mass of 38-43 kDa, was isolated from target cells susceptible to DNA fragmentation (Ucker *et al.*, 1992). This nuclease activity was not detectable in subclones of the target cell line resistant to CTL-induced DNA damage. It is unclear whether or not damage to the nuclear envelope

precedes DNA degradation following CTL attack (Howell and Martz, 1988; Duke and Sellins, 1989; Ucker *et al.*, 1992).

Often the terms apoptosis and preprogrammed cell death are used synonymously, but different control mechanisms are involved in each of these processes (Lockshin and Zakeri, 1991). Preprogrammed cell death is commonly observed during normal development and differentiation. In contrast, apoptosis is a series of morphological changes occurring within a cell. These events can be induced by a variety of stimuli, including those responsible for preprogrammed cell death. Further descriptions of necrosis, apoptosis, and preprogrammed cell death, as well as their occurrence and possible involvement in CTL-mediated lysis, can be found in a number of sources (Arends and Wyllie, 1991; Ellis *et al.*, 1991; Fawthrop *et al.*, 1991; Golstein *et al.*, 1991; Tomei and Cope, 1991; Cohen *et al.*, 1992; Gerschenson and Rotello, 1992).

Observations have been made to support, as well as to refute, both models. In support of the osmotic rupture theory, perforin alone can induce target cell death (Duke *et al.*, 1989; Shi *et al.*, 1992b). Lysis proceeds to the same extent in either the presence or absence of DNA fragmentation (Ucker *et al.*, 1992) and the nature of the target cell determines the type and degree of DNA damage (Sellins and Cohen, 1991). Perhaps the most significant observation in favor of this hypothesis is that CTL-mediated lysis does not require a metabolically competent target (Ratner and Clark, 1991). Furthermore, RNA and protein synthesis are not required for target cell lysis (Duke *et al.*, 1983); both are usually necessary for apoptotic cell death. Similarly, while apoptosis takes place over a period of several hours, CTL-induced DNA fragmentation occurs rapidly (Russell, 1983; Duke and Cohen, 1983).

In response to these arguments it has been proposed that CTL-mediated lysis bypasses the normal controls of apoptotic death. There may be a direct transfer of the inducer molecule(s) from the CTL to the target which may directly activate, or be responsible for, the final steps of the apoptotic pathway (Golstein *et al.*, 1991). However, there has been little success in detecting the transfer of potential inducers from the CTL to the target cell.

Delivery of the lethal hit by granule exocytosis

The most widely accepted model of lysis involves exocytosis of cytoplasmic granules from the CTL. Granule contents, including perforin and the CCPs, are released into the synapse between effector and target cell membranes. This type of vectorial release has several advantages over other mechanisms of delivery (Podack and Kupfer, 1991). For example, it ensures the highest concentration of effector molecules possible within a confined area. Directed release also limits the response to the appropriate target cell and protects labile proteins from inactivation by soluble inhibitors.

Ample evidence supports a granule exocytosis model. One reason that it was suggested is that both CTL-mediated lysis and exocytosis require calcium. Initial observations of granule translocation to the point of contact between the two cells provided the first direct evidence (Kupfer *et al.*, 1985). Inhibitors of exocytosis block granule release and prevent lysis of the target cell (Joag *et al.*, 1989). In addition, antibodies against granules prevent cytolysis by NK (Reynolds *et al.*, 1987). Granule proteins, including HF and proteoglycans, have been detected in the media following contact between CTL and target (Schmidt *et al.*, 1985; Tschopp and Nabholz, 1990). The lesions present in target cell membranes support the hypothesis that perforin is also released by exocytosis (Young, 1989; Krahenbuhl and Tschopp, 1991).

Isolated cytolytic granules, as well as purified granule proteins, induce target cell lysis with all the characteristics observed in CTL and NK mediated lysis (Henkart *et al.*, 1984; Millard *et al.*, 1984; Podack and Konigsberg, 1984; Masson *et al.*, 1985; Podack *et al.*, 1988). As mentioned above, perforin alone induces osmotic lysis of target cells with no DNA fragmentation. In the presence of sublethal doses of perforin, several members of the rat serine proteinase family termed fragmentins (species homologs of murine HF and the CCPs) are capable of inducing DNA fragmentation within target cells (Shi *et al.*, 1992a; 1992b). Fragmentin 2, the homolog of murine CCP1, produces DNA damage within 2-4 hours, while considerably longer incubation periods are required to see this effect with Fragmentins 1 and 3. Thus, perforin

and the CCPs provide the components required to induce the same type of death as that observed with intact CTL or NK.

Granule-independent lysis

Granule exocytosis, as measured by the release of BLT esterase activity into the media, can be prevented by conducting the lytic assays in the presence of EGTA to chelate extracellular calcium (Ostergaard *et al.*, 1987; Trenn *et al.*, 1987). However, even in the absence of perforin and CCP release CTL can efficiently lyse target cells (Young *et al.*, 1987). The lack of correlation between the amount of granule release and the extent of lysis (Clark *et al.*, 1988; Ratner and Clark, 1991) suggests that alternate mechanisms are also important during lysis.

Perhaps the most convincing argument in favor of granule-independent lysis is provided by PEL. These cells have no detectable granules, hemolytic activity, or BLT esterase activity, yet they can efficiently lyse their cognate targets(Berke and Rosen, 1987; 1988; Dennert *et al.*, 1987).

The effector molecules utilized in these circumstances have not been characterized. However, as discussed below, a number of potential mediators have been proposed. One, or a combination, of these may be involved.

Calcium-induced target cell lysis

A rapid increase in cytosolic calcium levels occurs within the target cell immediately after the association with a CTL. Tirosh and Berke (1985) proposed that this increase, resulting from both influx and release from intracellular stores, is sufficient to induce target cell lysis. According to their model, increased calcium activates endonucleases and topoisomerases responsible for DNA fragmentation and unwinding respectively. A sustained increase in calcium also stimulates cellular proteinases which participate in membrane damage. Death results from osmotic lysis induced by inactivation of ion pumps and from internal disintegration (Berke, 1991).

This model is not entirely in disagreement with the granule-exocytosis proposal since granules induce similar types of changes in the target cell and both mechanisms are dependent upon calcium.

Soluble mediators

The molecules which function as soluble mediators of lysis were discussed earlier. They include TNF∝, LT, leukalexin, and NKCFs. Each of these molecules induces DNA fragmentation in target cells (Schmid *et al.*, 1986; Liu *et al.*, 1987; Wright and Bonavida, 1987; Vassali, 1992). However, several factors argue against a primary role for any or all of these molecules in CTL, NK, or PEL mediated lysis.

The cytolytic activity of these proteins is neither calcium-dependent nor antigen specific. Furthermore, lysis induced by these molecules is not contact dependent. In contrast, CTL-mediated killing is antigen-specific and dependent upon intimate cellular contact in the presence of calcium. The most convincing argument against a primary role is that the soluble mediators require several hours, and even days, to destroy their targets, while CTL-mediated DNA fragmentation is observed within an hour and lysis can be complete in less than 4 hours. Supporters for the involvement of soluble factors suggest that a directed release toward a damaged target cell membrane may increase the kinetics of action (Ruddle, 1985; Ortaldo *et al.*, 1987). However, further study is required to substantiate this theory.

Secretion of ATP as a lytic molecule

Filippini *et al.* (1990a, 1990b) demonstrated that contact between effector and target cells results in the accumulation of high levels of ATP in the intercellular space. They proposed that ATP associates with receptors and kinases on the target cell surface and that this interaction produces numerous intracellular events which ultimately lead to target cell death. Extracellular ATP can induce apoptosis in certain cells (Zheng *et al.*, 1991). However, susceptibility to lysis and DNA fragmentation depends upon the nature of the target cell (Zanovello *et al.*, 1990). The validity of this model remains to be determined.

In support of this proposal, ATP can induce changes in ion permeability which interfere with sodium, potassium, and calcium homeostasis. A number of studies utilizing various ionophores indicate that membrane disruptions can induce increases in intracellular calcium (Albritton, 1988b; Kraut *et al.*, 1990; Ojcius *et al.*, 1991a). Thus, ionic changes lead to DNA fragmentation and lysis in a time frame similar to that seen with intact CTL. Whether these perturbations are induced by membrane contact (as suggested by Tirosh and Berke, 1985) or by extracellular ATP (Filippini *et al.*, 1990a) requires further analysis.

Resistance of CTL to lysis

One of the mysteries surrounding CTL-mediated lysis is how the CTL destroys the target cell without destroying itself. This point has been somewhat contentious. While CTL are susceptible to complement-mediated lysis, they are resistant to lysis by other CTL, by lytic granules, and by purified perforin (Blakely *et al.*, 1987; Kranz and Eisen, 1987; Verret *et al.*, 1987; Nagler-Anderson *et al.*, 1988; Shinkai *et al.*, 1988). However, under certain circumstances CTL can kill one another (Kranz *et al.*, 1987; Schick and Berke, 1990; Walden and Eisen, 1990; Moss *et al.*, 1991).

Several theories attempt to explain the protection of CTL from their own lytic machinery. One model proposes that the proteoglycans associated with perforin and the CCPs in the granules provide a protective barrier between the lytic molecules and the CTL membrane (Tschopp and Masson, 1987; Bleackley *et al.*, 1988b). Alternatively, several proteins are postulated to protect a CTL from lysis. An integral membrane protein called homologous restriction factor provides protection against both complement and LGL-mediated lysis (Tschopp and Nabholz, 1990; Podack *et al.*, 1991). Expression of this protein increases following stimulation with αCD3 mAb suggesting that protection is enhanced as the level of cytolytic potential develops. Cell-surface ecto-ATPase activity may protect the CTL from increased concentrations of extracellular ATP (Filippini *et al.*, 1990a). A third protein on CTL

membranes appears to bind perforin, thus protecting the CTL from osmotic lysis (Jiang *et al.*, 1990). Although any or all of these mechanisms may be involved in protection, further investigation is required to clarify this controversy.

INHIBITORS OF CTL-MEDIATED LYSIS

Numerous inhibitors have been utilized to examine the mechanism of CTL-mediated lysis. These studies focused on the role of exocytosis as well as that of the serine proteinases in the lytic process. The types of inhibitors used and the results obtained are summarized in the following section.

General inhibitors

Early studies involved the use of a variety of inhibitors to elucidate the process of CTL-mediated lysis. For example, several agents block cytolysis by interfering with the adhesion between effector and target. The list includes colchicine, cytochalasin B, dimethylsulfoxide, mercaptoethanol, and compounds such as dibutyryl cAMP which interfere with cAMP metabolism (Gately and Martz, 1977; Gately *et al.*, 1980; Hiserodt *et al.*, 1982b). A few, including prostaglandin E_2 and some cAMP modulating agents, also affect the programming and KCIL phases of lysis (Martz, 1975; Hiserodt *et al.*, 1982b).

A number of inhibitors which block exocytosis have also been used to study the lytic mechanism. Colchicine disrupts microtubule organization, chloroquine increases the pH of acidic granules, monensin interrupts membrane traffic through the Golgi apparatus, and inhibitors of energy metabolism have a variety of effects. All block CTL degranulation and inhibit cytolytic activity (Henkart, 1985; Joag *et al.*, 1989). Colchicine, as mentioned above, also interferes with adhesion. Cyclosporin A (CsA), an immunosuppressive agent, prevents degranulation with no effect on phosphatidyl inositol metabolism or calcium fluxes (Trenn *et al.*, 1989; Hultsch *et al.*, 1990). CsA also blocks the cytolytic effector activity of certain cloned CTL lines (Havele and Paetkau, 1988; Paetkau *et al.*, 1988).

Apoptosis is dependent upon both transcription and translation. Therefore, inhibitors of protein and RNA synthesis were used to examine the role of apoptosis in CTL-mediated lysis. Neither actinomycin D nor cycloheximide blocked the lysis or DNA fragmentation induced in the target cell by $CD8^+$ CTL (Ju, 1991). Both zinc, which inhibits the calcium and magnesium dependent endonuclease, and EGTA, which chelates calcium, blocked DNA fragmentation. However, caution is required in interpreting the effects of such nonspecific agents.

These experiments have revealed a great deal regarding the processes involved in delivery of the lethal hit by CTL. They have pointed toward a role for both exocytosis and DNA fragmentation in target cell lysis. Further clarification of these processes requires the development of more specific inhibitors.

Serine proteinase inhibitors

Identification and isolation of the CTL-specific granule serine proteinase family stimulated research into their involvement in cytolysis. Early studies relied on a number of relatively nonspecific inhibitors. For example, tosyl lysine chloromethyl ketone and tosyl phenylalanine chloromethyl ketone, inhibitors of trypsin and chymotrypsin respectively, were shown to block target cell lysis (Chang and Eisen, 1980; Redelman and Hudig, 1980). Both of these agents were subsequently shown to interfere with calcium influx (Utsunomiya and Nakanishi, 1986), therefore questioning the validity of previous observations. Purified HF is susceptible to a number of substances including diisopropyl fluorophosphate (DFP), and phenyl methyl sulfonyl fluoride (PMSF) (Ferguson *et al.*, 1988; Munger *et al.*, 1988; Hayes *et al.*, 1989). Although these agents inhibit enzyme activity and DNA fragmentation, they do not prevent lysis.

A major advancement was made with the identification of 3,4-dichloroisocoumarin (DCI) as a potent inhibitor of serine proteinases (Harper *et al.*, 1985). It is a general, mechanism-based inhibitor which is activated only when cleaved by a serine proteinase. This property confers both specificity and irreversibility. DCI blocks the activities

of all of the granule proteinases, although with different efficiencies. Most importantly, this inhibition occurs with no significant effect on the activity of perforin (Shi *et al.*, 1992b). It also prevents granule-mediated lysis and inhibits the components of the complement cascade (Hudig *et al.*, 1991; Kam *et al.*, 1992). Substituted isocoumarins have now been developed to provide greater inhibition of specific proteinases (Odake *et al.*, 1991). These compounds will be useful tools in elucidating the roles of each of the proteinases in CTL-mediated lysis.

The serine proteinase inhibitors have pointed toward the involvement of these proteins in CTL-mediated DNA fragmentation. Other possible roles have not yet been elucidated. Further characterization of the CCPs, as well as discovery of their *in vivo* substrates, will make it possible to clarify their role in cytolysis.

THESIS OBJECTIVES

The primary objective of the research presented in this thesis was to develop a greater understanding of the processes involved in CTL-mediated target cell lysis. As indicated in the preceding sections, many aspects of this complicated system remain to be clarified.

Several questions were asked to reach this objective:

1) Are cytolytic hybridomas capable of efficient target cell lysis without expression of mRNA for perforin and the CCPs? If so, what is involved in the lytic process?
2) What is the role of DNA fragmentation in CTL-induced cell death?
3) How are the CCPs involved in lysis?

The results presented in the following chapters provide new insight into the mechanisms of lysis utilized by CTL to destroy their targets. The final chapter provides a model based upon the findings of this research. In addition, questions which remain to be answered, along with possible approaches to address them, are presented.

CHAPTER 2: MATERIALS AND METHODS

Chemicals and reagents

Radiochemicals ^{51}Cr (in the form of Na_2CrO_4), $^{125}IUdR$, ($\alpha^{32}P$) deoxycytidine 5'-triphosphate tetra-(triethylammonium) salt ($\alpha^{32}P$-dCTP), ^{3}H-thymidine, ^{3}H-leucine, ^{35}S-methionine, and ^{3}H-Diisopropyl fluorophosphate (DFP) were obtained from New England Nuclear (NEN), Lachine Quebec.

The following chemicals were obtained from Sigma Chemical Co., St. Louis, MO., and stored in solution at -20°C unless otherwise indicated. Cycloheximide and Concanavalin A (ConA) were stored as aqueous solutions. 3,4-dichloroisocoumarin (DCI) was stored as a 10 mM stock solution in dimethylsulfoxide (DMSO). N-acetyl-L-tyrosine ethyl ester (ATEE) and N-benzoyl-L-arginine ethyl ester (BAEE) were prepared as 10 mM stock solutions in redistilled ethanol. Nα-benzyloxy carbonyl-L-lysine thiobenzyl ester (BLT), dissolved in water, and 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB), dissolved in dimethyl formamide, were also stored at -20°C.

Nicotinamide was prepared fresh daily as an aqueous solution. 4-amino-1,8-naphthalimide (ANA), dissolved in methanol, and (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), dissolved in phosphate buffered saline (PBS), were also prepared as needed. Cyclosporin A (CsA) was a gift from Sandoz Canada Inc., Dorval Quebec. Aurintricarboxylic acid (ATA), obtained from Aldrich Chemical, was dissolved in RPMI 1640 (Gibco BRL) supplemented with 20 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES, pH 7.4) and stored at 4°C.

Monoclonal hamster anti-murine CD3ϵ antibody (αCD3 mAb) was partially purified from the culture supernatant of hybridoma 145-2C11 by centrifugation, extensive dialysis against PBS at 4°C, and sterilization by irradiation with 5000 rads (Leo *et al.*, 1987). Aliquots were stored at -20°C. Recombinant murine TNF (rMuTNF), with a specific activity of 1.2×10^7 U/mg, was obtained from Genentech (San Francisco, CA.).

Cell culture

All cells were maintained in a 5% CO_2 humidified incubator at 37°C. Mixed lymphocyte culture (MLC) hybridomas MD90, MD45, and MD45-27J, as well as peritoneal exudate lymphocyte (PEL) hybridomas PN37 and PMM-1, generated in the lab of Gideon Berke as described previously (Kaufmann *et al.*, 1981), were grown by continuous culture in either Dulbecco's Modified Eagle's (DME) medium (Gibco) or RPMI 1640 (Gibco) supplemented with heat-inactivated 10% fetal bovine serum (HyClone), 20 mM HEPES, 100 μM 2-mercaptoethanol, 100 μg/ml streptomycin, and 100 IU/ml penicillin (DMEFM or RHFM respectively).

MTL 2.8.2, a T cell clone expressing perforin and the cytotoxic cell proteinases (CCPs) constitutively, was cultured in RHFM containing 30 U/ml recombinant human IL2 (rhIL2) (Barr *et al.*, 1984). EL4(HP2), a thymoma subcloned for high levels of inducible IL2 production, EL4, P815 (a mastocytoma cell line), A1.1 (a T cell hybridoma; Shi *et al.*, 1990), hybridoma 145-2C11 which secretes αCD3 mAb, and YAC-1 (in the absence of mercaptoethanol) were grown by continuous culture in RHFM. L929-8 fibroblast cells (Branch *et al.*, 1991) were maintained in Iscove's modified Dulbecco's medium (Gibco) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (Gibco) and 0.05 μg/ml gentamycin.

CTL 21.9(I) (Havele *et al.*, 1986) is a cloned $H2^k$ cytotoxic T cell line which requires antigenic stimulation (irradiated $H2^d$ BALB/c splenocytes at a ratio of 10-20:1 for 3 days) and 30 U/ml rhIL2 for activation and proliferation. Activated CTL were separated from antigen by centrifugation over 10% Ficoll 400 (Pharmacia)-10% metrazoic acid (Sigma) at 600 x g for 20 minutes at room temperature. CTL21.9(IA) is an adherent subclone of CTL 21.9(I) which demonstrates high levels of cytolytic activity following 24 hour stimulation with a 1:500 dilution of αCD3 mAb. All growth and stimulation conditions for this clone were similar to those of CTL21.9(I).

Activation of cytolytic cells

BALB/c and CBA/J mice, 6-8 weeks of age, were obtained from either the University of Alberta Laboratory Animal Services or from Jackson Laboratories. Spleen cells were isolated to be used as antigen or to be stimulated for the generation of CTL.

CTL were generated by three different methods: mixed lymphocyte reaction (MLR), ConA activation, or ∝CD3 mAb stimulation. For MLR, $1x10^6$ CBA/J responder cells ($H2^k$) were cultured with $1x10^6$ irradiated BALB/c ($H2^d$) stimulator cells per ml RHFM containing 20 U/ml rhIL2 for 4-6 days. ConA stimulation involved culture of $1x10^6$ CBA/J splenocytes per ml RHFM containing 20 U/ml rhIL2 and 10 μg/ml ConA for 3 days. Stimulation with ∝CD3 mAb involved culture of 0.5-$1x10^6$ BALB/c splenocytes per ml RHFM containing 20-30 U/ml rhIL2 with a 1:500 dilution of the antibody for 48-72 hours.

Induction of cytolytic activity in the PEL and MLC hybridomas was achieved by culturing cells at a density of $2x10^5$/ml for 18-22 hours in the presence of various stimuli: 2 μg/ml ConA; irradiated EL4(HP2) or EL4 cells at a 1:1 ratio; 500 U/ml rhIL2; or 500 U/ml rhIL2 + 2μg/ml ConA.

Assessment of cytolytic activity

Cytolytic activity was assessed by measuring the release of ^{51}Cr from labelled target cells (P815, EL4(HP2), EL4, or L929-8). In all cases, targets in the log phase of growth were incubated with 100 μCi ^{51}Cr per $2x10^6$ cells in a 37°C water bath for 60-90 minutes. Cells were then washed with 3 x 10 ml aliquots of RHFM, counted, and resuspended to a density of $1x10^5$ per ml.

CTL and labelled targets were incubated together at various effector to target (E:T) ratios in a total volume of 200 μl in 96-well V-bottom plates for four to six hours at 37°C in the presence or absence of various inhibitors. In certain assays ConA was added to a final concentration of 2 μg/ml. A 100 μl portion of the cell-free supernatant was removed for counting. CTL activity was determined using the formula: % lysis = 100 x (sample - spontaneous release)/(total -

spontaneous release). Spontaneous release was determined by incubation of target cells in the absence of CTL and lysis of target cells in 1% Zap-Isoton (Coulter) detergent was used to assess total release.

The cytolytic activity of rMuTNF on EL4 cells was measured in a similar manner using doubling dilutions of TNF with a constant number of target cells.

Determination of bystander lysis was achieved by incubating equivalent numbers of unlabelled appropriate targets and labelled inappropriate targets (ie, L929-8 or P815) with hybridoma effectors at various E:T ratios in the presence of ConA. Quantitation of lysis was performed as described above.

In certain experiments effectors, targets, or both were pretreated with various agents prior to addition in the chromium release assay. In all cases, incubations were carried out at 37°C for the times indicated in the figure or table legends. Removal of the pretreatment agent was carried out by 2 or 3 washes in 50 ml RHFM.

Quantitation of DNA fragmentation

The quantitation of DNA fragmentation was adapted from the method of Duke and Sellins (1989). Briefly, P815 or EL4 cells were labelled for 16-20 hours with ^{125}IUdR (2 μCi/ml). Following extensive washes in RHFM, $1x10^5$ targets were incubated with $1x10^6$ effectors in a final volume of 1.0 ml in Eppendorf tubes. The assay was terminated by centrifugation of the cells and resuspension in 1.0 ml lysis buffer (5 mM Tris-HCl, pH 8.0, 2 mM EDTA, 0.5% iton X-100). Cells were vigorously vortexed and incubated at 4°C for 20 minutes. Finally, nuclei were pelleted by centrifugation for 20 minutes at 14,000 x g. The supernatant, containing fragmented (soluble) DNA present in the cytoplasm, and the nuclear pellet (resuspended in 1.0 ml lysis buffer) were quantitated by gamma counting. Percentage DNA fragmentation was calculated as 100 x (soluble DNA/soluble + sedimented DNA). Background release, subtracted from both measurements, was determined by incubation and processing of targets alone.

Programming for lysis: Chromium release assay

In certain experiments P815 targets were programmed for lysis by incubation with adherent CTL21.9(IA), stimulated with 1:500 αCD3 mAb for 24 hours, in a 200 μl volume in 96-well flat-bottom plates. Conjugation and programming was allowed to proceed for 5 to 60 minutes at 37°C. A 100 μl aliquot of nonadherent target cells was removed, transferred to prewarmed plates containing 100 μl RHFM per well (with or without various inhibitors), and monitored for various lengths of time in a 200 μl volume in 96-well V-bottom plates. Supernatants (100 μl) were collected and quantitated for ^{51}Cr release as described above.

In later experiments, presented in Chapter 6, CTL 21.9(IA) effectors were set up at $5x10^5$ per well in 12-well cluster dishes for the assay. Programming was initiated by the addition of $5x10^4$ P815 targets. At the conclusion of the assay, the entire volume of targets was removed into Eppendorf tubes, gently mixed, and dispersed in 100 μl aliquots into 96-well V-bottom plates, prepared as described above, for determination of chromium release. Treatment of the targets in this manner reduced the variability amongst replicates in the assays.

Programming for lysis: Fragmentation assay

Fragmentation induced during the programming assay was assessed by incubating $1x10^5$ P815 targets, labelled as described above, with $1x10^6$ αCD3 mAb-activated CTL 21.9(IA) in a final volume of 0.5 mls in 6-well cluster dishes. Target cells were harvested into Eppendorf tubes and incubated in a 1.0 ml volume, in the presence or absence of various agents, for the times indicated in the various experiments. Fragmentation was measured as described above.

Programming for lysis: Assessment of target cell viability

Target cell viability was measured by incubation of unlabelled P815 cells with CTL 21.9(IA) as described for DNA fragmentation above. Once the cells were pelleted, the supernatant was discarded and targets were resuspended in 700 μl of fresh RHFM. Six aliquots (100 μl each) were distributed into 96-well flat bottom plates and incubated overnight

at 37°C. ^{3}H-thymidine (1 μCi per well) was added and incubations were continued for a further 8-10 hours. Cells were harvested with a Skatron Titertek Cell Harvester (Flow Laboratories) and incorporation was quantitated by liquid scintillation counting.

Alternatively, viability was determined utilizing the MTT assay described by Mosmann (1983). Briefly, 10 μl of MTT, dissolved to a concentration of 5 mg/ml in PBS and filter sterilized, was added to each well. Plates were mixed and incubated at 37°C for four hours. 20 μl of a 10% sarkosyl solution was added to each well, plates were mixed and incubated for a further 30 minutes at 37°C. Acid-isopropanol (80 μl of 0.04N HCl in isopropanol) was then added and plates were mixed vigorously on an IKA-Schuttler MTS4 plate mixer for 15 minutes. The plates were read on a Dynatech MR600 Microplate reader using a test wavelength of 570 nm and a reference wavelength of 630 nm.

Visualization of DNA fragmentation by agarose gel electrophoresis

Analysis of DNA fragmentation by electrophoresis was accomplished using a modification of the method of Smith *et al.* (1989). $1x10^{6}$ CTL 21.9(I) effector cells were mixed with $1x10^{6}$ target (P815) cells in a final volume of 1 ml RHFM in the presence or absence of 200 μM ATA. Cells were collected at various times and pelleted in Eppendorf tubes for 10 seconds. The pellets were resuspended in 35 μl lysis buffer (10mM EDTA, 50 mM Tris-HCl, pH 8.0 containing 0.5% (w/v) sodium lauryl sarkosinate and 0.5 mg/ml proteinase K) and incubated at 50°C for one hour. RNase A was added to a final concentration of 0.5 mg/ml and incubation was continued for one hour. Samples were electrophoresed in 1.0% agarose gels in Tris-Borate-EDTA buffer and DNA was visualized by UV following ethidium bromide staining.

Assessment of DNA fragmentation in isolated nuclei

A1.1 T-hybridoma cells (2.5×10^{7}) were labelled with 1 μCi/ml ^{125}IUdR for 16 hours. Cells were washed three times with ice cold PBS and then lysed with 5 ml ice cold isolation buffer (10 mM Tris, 2 mM $MgCl_2$). Samples were maintained on ice prior to assay.

The fragmentation reaction was carried out in Eppendorf tubes for 130 minutes at 37°C. Each tube contained 0.1 ml of nuclei in the presence or absence of 50 mM EDTA or 100 μM ATA. Reactions were terminated by the addition of 1 ml 10 mM Tris, 30 mM EDTA, and 0.5% Triton X-100 to each tube. After vortexing, samples were centrifuged and both the supernatant and sediment were collected for determination of radioactivity in a gamma counter.

RNA isolation

RNA was isolated using guanidinium thiocyanate solubilization and centrifugation through cesium chloride according to the method of Chirgwin *et al.* (1979).

Northern blots were prepared by denaturing total RNA (10-15 μg) in two volumes of FFMOPS [50% formamide, 6.5% formaldehyde, 1x MOPS buffer (consisting of 20 mM MOPS, 5 mM sodium acetate, 1 mM EDTA, pH 7.0)] at 60°C for 15 minutes. Tracker dye was added and RNA was size-fractionated by electrophoresis in 1% agarose gels containing 0.7% formaldehyde and 0.33 μg/ml ethidium bromide. Integrity of the RNA was assessed by UV visualization of the ribosomal RNA bands. Finally, the RNA was transferred onto Hybond N nylon filters (Amersham) by placing the filter in contact with the gel under weight overnight. The membranes were cross-linked in a UV Stratalinker™ 1800 using the auto-crosslink setting.

Polymerase chain reaction (PCR) analysis of perforin and cytotoxic cell protease (CCP) expression

Quantitative PCR analysis of the CCPs was carried out essentially as described previously (Prendergast *et al.*, 1992b).

cDNA was synthesized after denaturing 1 μg of each RNA sample and 100 ng of primer at 94°C for 2 minutes. Samples were chilled on ice and reagents added in the following order: 50 mM Tris-HCl (pH 8.3); 40 mM KCl; 6 mM $MgCl_2$; 10 mM DTT; 1 μg BSA; 2.5 units RNasin (Promega); 0.5 mM dNTPs and 200 units M-MLV reverse transcriptase (BRL). After incubation at 37°C for 1 hour samples were subjected to PCR analysis.

A 100 μl PCR reaction contained 10 mM Tris (pH 8.3); 50 mM KCl; 0.01% gelatin; various concentrations of $MgCl_2$; 50 μM dNTPs; 2.5 units of Taq polyermase (BMC or BRL); 50 nM of each primer and either control DNA or cDNA derived from 0.1 μg of RNA. PCR involved an initial 3 minute 94°C denaturation step followed by 35 cycles on a Hybaid thermocycler (Bio/Can Scientific). Each PCR cycle consisted of a 1 minute denaturation step (94°C), a 1.5 minute annealing step, and a 2.5 minute extension step (72°C).

Each transcript was detected using a specific primer set, with the exception of the CCP3 to CCP5 gene transcripts which could be detected using a single primer set. Primer sequences and the expected PCR product sizes are listed in Table 2.1. The optimal annealing temperatures and $MgCl_2$ concentrations for each primer set are also listed.

Two controls were included in each experiment for determination of cDNA synthesis and PCR efficiency. First, MTL 2.8.2 RNA was reverse transcribed and PCR amplified with the reagents and conditions used to prepare hybridoma RNA samples. This control ensured that the cDNA synthesis step was effective. Secondly, a dilution series of reverse transcribed MTL 2.8.2 RNA and plasmid DNA was PCR amplified to determine the detection limit of each PCR series.

Aliquots of each PCR reaction were electrophoresed in 1.5% agarose gels at 125 volts for 2 hours. DNA was visualized with UV following ethidium bromide staining. The DNA products were denatured, neutralized, and transferred onto Hybond N nylon filters (Amersham).

Full-length mouse perforin cDNA (generated by PCR amplification) and cDNA inserts of the various CCPs were radioactively labelled to a specific activity of approximately 1 x 10^8 cpm/μg in a modified random primer reaction (Feinberg and Vogelstein, 1983) using Klenow fragment (BRL) and α^{32}P-dCTP. The reaction involved denaturation of up to 1 μg of DNA in water for 3 minutes at 95°C. The sample was cooled briefly and the following components were added to achieve a final volume of 50 μl: 10 μl 5x reaction buffer, 20 μg BSA (Boehringer Mannheim), 200 mCi α^{32}P-dCTP, and 3U Klenow. Samples were incubated overnight at room

Table 2.1: Primer sequences, predicted product sizes, and optimal conditions for detection of actin, perforin, and CCP1-5.

Transcript	Primers	Product Size (bp)	Optimal Annealing Temp (°C)	Optimal $MgCl_2$ Concentration (mM)
CCP1	CCP1 - CCP1/2	389	45	1.2
CCP2	DAS - CCP1/2	439	45	1.2
CCP3	DAS - UE5	659	45	1.2
CCP4	DAS - UE5	659	45	1.2
CCP5	DAS - UE5	659	45	1.2
PERFORIN	Per-N - Per-C	362	58	0.6
ACTIN	Clontech*	500	60	0.75

Primer sequences for reverse transcription were as follows: CCP1: AGGATCAGCAGCCTGAG; CCP1/2: GATCCTTCTGTACTGTCA; DAS: TCCTGATTCTCCTGACC; UE5: GCTTATCCACGGCAGG; Per-N: CCACGACAGAGGGGCTTGG; Per-C: CCACTGCGGTTTCCTGGAGG.

* Primers for mouse β-actin were purchased from Clontech.

temperature. The 5x reaction buffer was prepared by combining 100 parts of a solution containing 0.5 mM each of dATP, dTTP, and dGTP in 1.25 M Tris-HCl, 0.125 M $MgCl_2$, pH 8.0, 0.25M 2-ME with 250 parts 2 M HEPES, pH 6.6 and 150 parts hexadeoxyribonucleotides [$pd(N)_6$ obtained from Pharmacia] at 90 ODU/ml in tris-EDTA buffer. Unincorporated label was removed by filtration through a column of Sephadex G-50 (medium) equilibrated in tris-EDTA buffer, pH 8.0.

Blots were incubated for 5 hours in prehybridization buffer consisting of 6xSSC (1xSSC: 15 mM sodium citrate; 150 mM NaCl, pH 7.0), 5x Denhardt's (100x Denhardt's: 1% Pharmacia Type 400 Ficoll, 1% polyvinylpyrrolidone, 1% BSA), 0.5% SDS, and 20% formamide. Hybridizations were carried out overnight in the same buffer. Blots were washed to a final stringency of 0.2xSSC-0.1%SDS at 60°C and DNA bands were visualized by autoradiography onto Kodak X-OMAT film.

Bioassay of TNF activity

Determination of TNF activity, representing both TNFα and LT, in the supernatant, cytosolic, and membrane fractions of activated PEL and MLC hybridomas, as well as EL4 cells, was performed as described by Lapchak *et al.* (1992) using L929-8 fibroblast cells as indicators.

Detection of ^{3}H-DFP binding proteins

The induction of DFP-binding activity was examined by incubation of hybridoma effectors ($1x10^6$) with an equal number of EL4 targets ($1x10^6$) at 37°C for various periods of time. CTL or EL4 alone, at a number equivalent to the total, were used as controls. Cells were harvested, and washed by centrifugation in PBS. The pellets were prepared for assay of ^{3}H-DFP binding activity according to the method of Masson *et al.* (1986a).

Equivalent volumes of each reaction were mixed with 2 volumes of Laemmeli sample buffer containing 0.1 mM DTT. Samples were boiled at 95°C for 5 minutes and loaded onto a 12% SDS-polyacrylamide gel. Gels were fixed in a solution of 10% methanol - 10% acetic acid, soaked in

Enlighten (NEN) for approximately 15 minutes, and dried. Labelled proteins were detected by autoradiography onto Kodak X-OMAT film.

Perforin and Fragmentin: Purification and assay of activity

Perforin and fragmentin were purified in the laboratory of Dr. A. H. Greenberg, University of Manitoba as described previously (Shi *et al.*, 1992b). YAC-1 target cells were double-labelled for 60-90 minutes with both ^{51}Cr and $^{125}IUdR$, washed, and set up at the appropriate density in 96-well assay plates for assessment of both chromium release and fragmentation. Various concentrations of perforin and/or fragmentin were added and incubations were carried out for the periods of time indicated. Plates were centrifuged and aliquots were counted to determine release of both labels. Triton X-100 was used for quantitation of DNA fragmentation.

Determination of BLT esterase activity

Assessment of BLT esterase activity employed a procedure which was modified from that of Green and Shaw (1979). Briefly, $5x10^6$ MLR-induced CTL were incubated with $1x10^5$ P815 stimulator cells $\pm$ ATA for 4 hours at 37°C in Hanks Balanced Salt Solution (Gibco) supplemented with 0.01M HEPES and 1 mg/ml BSA in 96-well round bottom plates. Supernatants (50 μl) were transferred to 96-well flat-bottom plates containing 50 μl BLT and 100 μl DTNB per well. Plates were mixed and the color was allowed to develop for various amounts of time at room temperature. OD readings were taken at 410 nm using the Elisa platereader. Spontaneous release was detected by incubation of effectors or targets alone, while total release represents the amount of activity detected in supernatants of effectors lysed with Zap detergent.

Programming for lysis: Assessment of BLT esterase release

Programming was initiated by adding $1x10^4$ P815 targets to $1x10^5$ CTL 21.9(IA) stimulated overnight with αCD3 mAb in 96-well flat-bottom plates. Following incubation for the various times, plates were centrifuged briefly and 100 μl aliquots were transferred to wells containing 50 μl BLT and 100 μl DTNB. Determination of activity was carried out as described above.

CHAPTER 3: CYTOLYTIC HYBRIDOMAS, DEVOID OF PERFORIN AND THE CYTOTOXIC CELL PROTEINASES, UTILIZE A NOVEL LYTIC MECHANISM.

INTRODUCTION

CTL and NK destroy malignant and virally-infected cells. These effectors employ a variety of mechanisms to induce death in their targets and several molecules with the potential to function as lytic agents have been identified (reviewed by Henkart, 1985; Young, 1989; Tschopp and Nabholz, 1990). The most commonly accepted model of lysis, employed by both NK and CTL, involves granule exocytosis. According to this model, contact between effector and target is followed by a reorganization of the cytoskeletal components within the effector. This rearrangement brings the cytolytic granules containing perforin, as well as the cytotoxic cell proteinases (CCPs; also known as granzymes), into close proximity with the contact site between the effector and target. The contents of the granules are released in a controlled fashion into the space between the two cells. Lysis of the target is presumably mediated by the exocytosed granule proteins.

Perforin alone is capable of inducing target cell lysis (Duke *et al.*, 1989). However, not all of the observations concerning CTL-induced death can be explained by a mechanism involving perforin alone. For example, lysis is usually preceded by fragmentation of the nuclear DNA into an oligonucleosomal ladder, reminiscent of the degradation observed during apoptosis (Duke *et al.*, 1983; Russell, 1983). However, perforin alone is incapable of inducing this type of controlled DNA destruction (Duke *et al.*, 1989). The CCPs are also released during the degranulation event, but their role in the lytic process has not been clearly elucidated. Recent evidence with NK proteinases, however, suggests a role for these proteins in the fragmentation of cellular chromatin (Shi *et al.*, 1992a; 1992b). Although CTL may induce apoptosis in their targets, this process is not dependent upon the synthesis of new proteins as it often is with classical programmed cell death (Duke *et al.*, 1983; Ucker *et al.*, 1989; Odaka *et al.*, 1990; Shi *et al.*, 1990).

CTL-mediated lysis must therefore by-pass the normal control mechanisms involved in apoptosis.

The validity of the granule-mediated exocytosis model as a unifying hypothesis has been challenged by several observations. Studies indicate that CTL-mediated lysis of certain targets occurs at external calcium concentrations of less than 1μM - conditions at which granule exocytosis does not occur and perforin is not lytic (Tirosh and Berke, 1985; Ostergaard *et al.*, 1987; Trenn *et al.*, 1987). Perhaps the most convincing argument is the observation of effective lysis of targets by peritoneal exudate lymphocytes (PEL) in the absence of either perforin (hemolytic) or proteinase (BLT esterase) activity (Berke, 1987; Dennert *et al.*, 1987; Duke *et al.*, 1989). However, this observation is complicated by reports of perforin and CCP mRNA expression in these cells (Brunet *et al.*, 1987; Nagler-Anderson *et al.*, 1989; Garcia-Sanz *et al.*, 1990; Ebnet *et al.*, 1991; Griffiths and Mueller, 1991). Heterogeneity of freshly isolated PEL is one problem in these investigations. For example, contamination with NK may explain the presence of this mRNA. *In vitro* culture of primary PEL with IL2 induces accumulation of lytic granules containing perforin and the CCPs (Berke and Rosen, 1988). These granules may arise as a consequence of culture conditions rather than as a functional necessity.

Cytolytic hybridomas provide an attractive model for study since they consist of a pure cell population with inducible cytolytic activity (Kaufmann and Berke, 1983) and no requirement for IL2. These hybridomas were generated by polyethylene glycol-induced fusion of CTL or PEL with BW5147 lymphoma cells (Kaufmann *et al.*, 1981). These cells express the characteristic surface markers of CTL and lyse targets in an antigen-specific manner. The killing induced by the hybridomas is similar to that induced by CTL. For example, lysis is inhibited by trypsin and EDTA, and requires a temperature of 37°C.

Two PEL hybridomas, as well as three mixed lymphocyte culture (MLC) hybridomas, exhibiting varying levels of inducible cytolytic activity were examined for expression of perforin and CCP1-5 transcripts using the sensitive polymerase chain reaction (PCR). In addition,

studies to elucidate the pathway of killing utilized by two of these clones were performed. Experiments confirmed that these cytolytic hybridomas can efficiently lyse targets in the absence of either perforin or the family of proteinases CCP1-CCP5. Furthermore, lysis was dependent on protein synthesis and appeared to involve the enzyme poly(ADP-ribose)synthetase. A 16kDa protein with ^{3}H-diisopropylfluorophosphate (DFP) binding ability, most likely a serine proteinase, was induced during the lytic cycle. Lysis was partially inhibited by the general serine proteinase inhibitor 3,4-dichloroisocoumarin (DCI). Unlike conventional CTL-mediated lysis, DNA fragmentation accompanied, but did not precede, membrane disintegration. This degradation was not diminished, nor was target cell lysis prevented, by an endonuclease inhibitor previously shown to block both apoptosis and CTL-mediated death. Finally, it was demonstrated that the soluble lytic molecule TNF was not involved. These results suggested the existence of an alternate mechanism of target cell lysis.

RESULTS

PEL and MLC hybridomas exhibit cytolytic activity

The hybridomas used in this study exhibited varying levels of cytolytic activity against EL4(HP2) targets following antigenic or mitogenic (ConA) stimulation (Table 3.1). In contrast, unstimulated hybridomas displayed only background levels of cytolysis. High levels of rhIL2 were ineffective as a stimulus of cytotoxicity. Induction of cytolytic activity in hybridoma MD45-27J was not observed, regardless of the mode of stimulation.

Cytolytic hybridomas do not express perforin or CCP1-5 mRNA

Total cellular RNA was isolated from all hybridomas following a variety of different stimuli. Equivalent amounts of RNA (15 μg) were electrophoresed in denaturing agarose gels to determine sample integrity. Ethidium bromide staining revealed distinct ribosomal RNA bands in each lane. Furthermore, the intensity of these bands was approximately the same suggesting an equivalent load per lane. Gels

Table 3.1: Cytolytic activity of PEL and MLC hybridomas.

	Unstimulated	Antigen	ConA	IL2
PEL Hybridoma				
PMM-1	1.3	29.9	4.8	0
PN37	2.0	8.3	11.6	5.5
MLC Hybridoma				
MD90	4.0	17.7	17.3	2.4
MD45	1.0	21.4	11.9	ND
MD45-27J	0	1.4	3.5	3.2

All hybridomas were set up at a cell density of 2 x 10^5/ml for 18-22 hours with media only (unstimulated), irradiated EL4(HP2) at a 1:1 ratio (antigen), 2 μg/ml ConA, or 500 U/ml rhIL2. Results are expressed as percentage specific ^{51}Cr release from EL4(HP2) targets in a standard 4 hour assay in the presence of 2 μg/ml ConA at a 5:1 effector to target ratio.

ND = not determined.

were blotted and the Northerns were probed with cDNAs for the various proteinases (CCP1 - CCP5), as well as perforin, Granzyme A (HF), and lymphotoxin. Even when the autoradiograms were overexposed, no bands were detected in anything other than the MTL 2.8.2 positive controls (results not shown). These results suggested that if perforin or the CCPs were present in these cytolytic hybridomas, their levels of expression must be very low.

RNA samples were subjected to cDNA-PCR analysis (carried out by Dr. Prendergast) and reaction products were initially detected in the gels using UV after ethidium bromide staining. Actin primers were used as a positive control to ensure that all samples contained RNA that could be reverse transcribed and PCR-amplified. The top panel of Figure 3.1 shows the ethidium bromide-stained actin band observed in all samples.

With the exception of the MTL 2.8.2 RNA and the plasmid dilution series no bands of the appropriate size were observed by ethidium bromide staining using the perforin or CCP primers. Sensitivity of detection was enhanced by Southern blotting and hybridization using labelled cDNA insert probes. Results are shown in Figure 3.1. The limits of detection, calculated using the plasmid dilution series, were less than one transcript per cell for perforin and the CCPs.

No CCP1 transcripts were detected in any of the hybridomas following activation. CCP2 expression was also absent from stimulated, as well as unstimulated, hybridomas. Likewise, no perforin or CCP3-5 transcripts could be observed, even following overexposure of the autoradiographs (not shown). Expression of CCP1 and CCP2, corresponding to no more than 1 transcript per cell, was observed in an inconsistent fashion. For example, CCP1 was present in unstimulated MD90 and PN37 hybridomas, as well as in rhIL2-stimulated PMM-1 cells. CCP2 was detected in antigen-stimulated MD45 cells. In spite of these few examples of low level expression, development of cytolytic potential took place in the absence of CCP and perforin mRNA. EL4(HP2), the antigen and target for the hybridomas, expressed neither perforin nor proteinase transcripts.

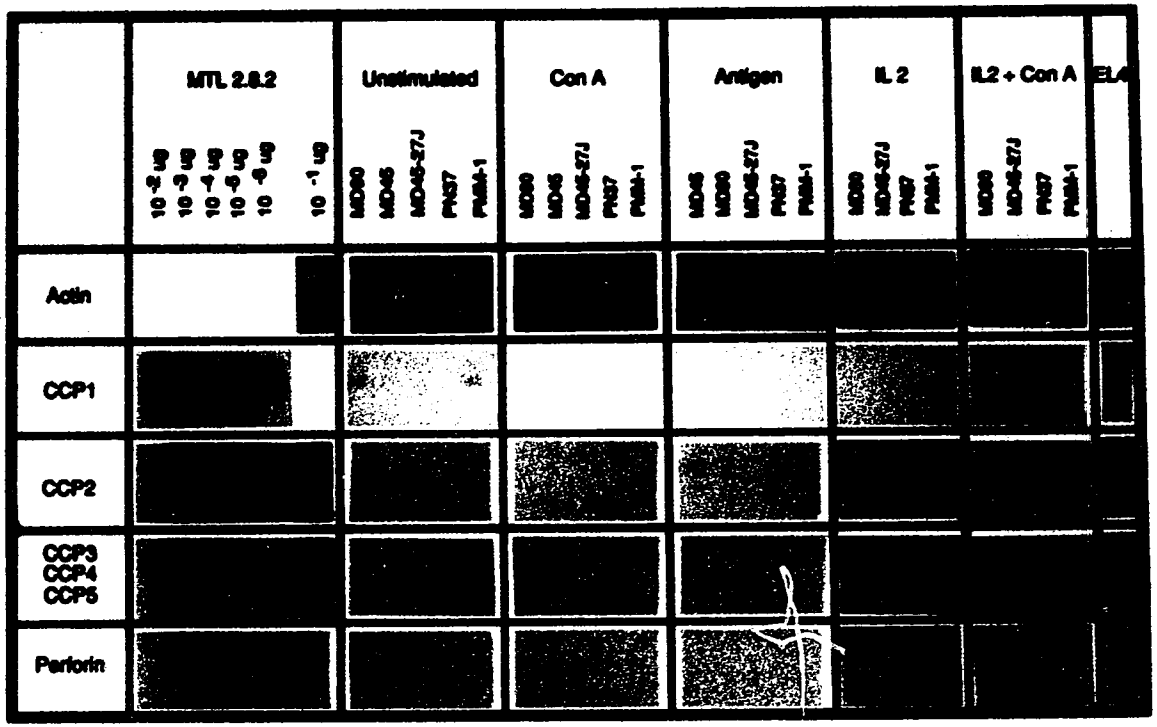


Figure 3.1: PCR analysis of PEL and MLC hybridoma RNA.

PCR analysis was carried out on total cellular RNA isolated from the hybridomas, as well as from the positive control T cell line MTL 2.8.2 and the target cell line EL4(HP2) as described in Chapter 2. PCR products were detected by ethidium bromide staining following gel electrophoresis (Actin) or by hybridization with radioactively labelled cDNA insert probes of Southern blotted DNA (CCP1-5 and perforin). A dilution series of the MTL 2.8.2 RNA serves as a reference for establishing the limits of detection.

Protein synthesis is required for target cell lysis

Target cell lysis mediated by CTL containing perforin and the CCPs is thought to occur without a requirement for protein synthesis (Duke *et al.*, 1983), although contrary results have been obtained (Zychlinsky *et al.*, 1991). $CD4^+$ CTL, on the other hand, do require protein synthesis for target cell lysis. The effect of cycloheximide, a protein synthesis inhibitor, on the lytic capabilities of two of the hybridomas was examined. Addition of cycloheximide to the lytic assay completely inhibited lysis mediated by the MLC-derived hybridoma MD90 (Figure 3.2). Although PMM-1 was less sensitive to inhibition of protein synthesis, a significant reduction in lysis was observed (i.e., approximately 50% - 80% inhibition at various E:T).

The continuous presence of cycloheximide during the interaction of the effectors and targets was required. Pretreatment of either the EL4 targets or the CTL effectors (not shown), followed by washing prior to inclusion in the assay, had no significant effect on the lysis mediated by either hybridoma. These results suggest that protein synthesis is triggered following the interaction between the two cells. It is not possible from these experiments to determine which cell is actively making the required proteins.

A serine proteinase is involved in lysis

Although CCP1 - CCP5 are not involved in the lytic pathway utilized by these CTL, we examined the effect of the general mechanism-based serine proteinase inhibitor DCI on cytotoxicity. This compound interacts specifically with the active site serine residue, resulting in inhibition of enzyme activity (Harper *et al.*, 1985). Surprisingly, a dose-dependent inhibition of chromium release was observed at all E:T ratios with both hybridomas (Figure 3.3). These results suggest the participation of an unidentified serine proteinase in target cell lysis.

Equivalent numbers of effectors and targets were mixed together and allowed to interact for various amounts of time. Cell lysates were prepared and incubated with ^{3}H-diisopropylfluorophosphate (DFP), a compound utilized for detection of serine proteinase activity. Several

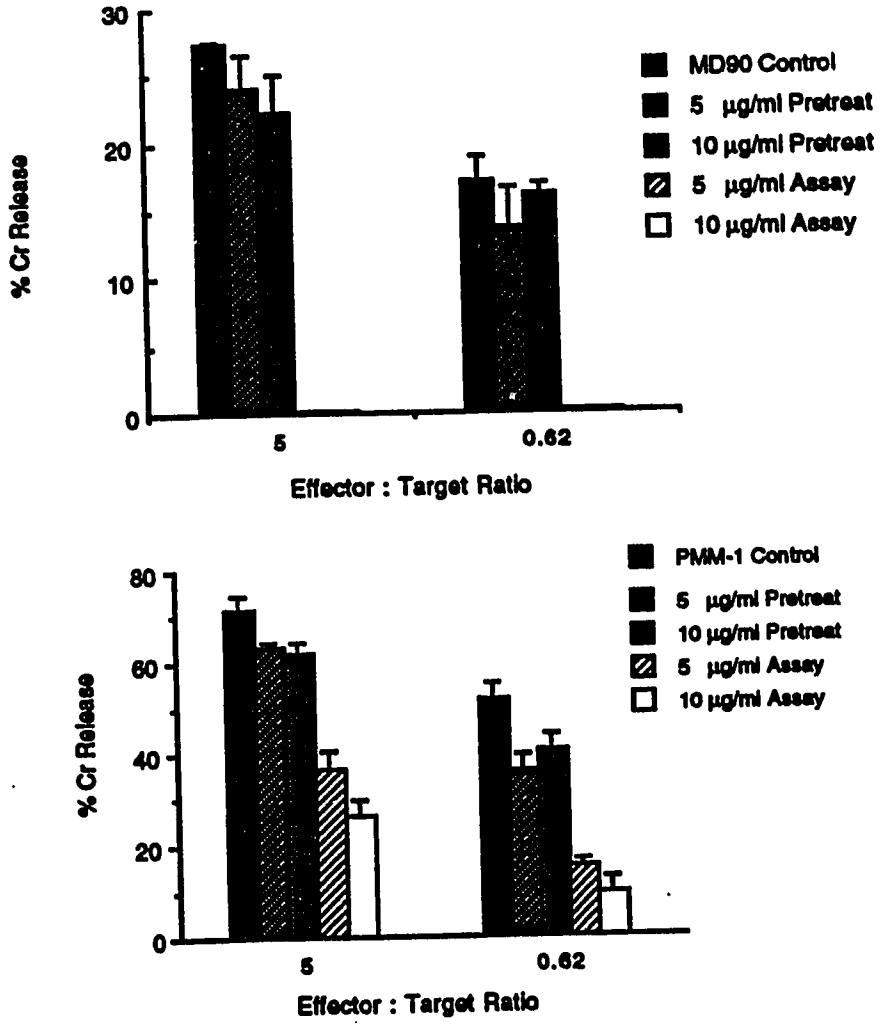


Figure 3.2: Cycloheximide inhibits cytolysis by MD90 and PMM-1. MD90 and PMM-1 effectors were antigen-stimulated for 18 hours prior to assay. EL4 targets were pretreated with cycloheximide at 37°C for 1 hour. Cycloheximide was either present during the assay (Assay) or removed by washing the targets twice with RHFM prior to addition (Pretreat). Assays were carried out as described in Chapter 2. Results are expressed as the mean ± SD of triplicate determinations.

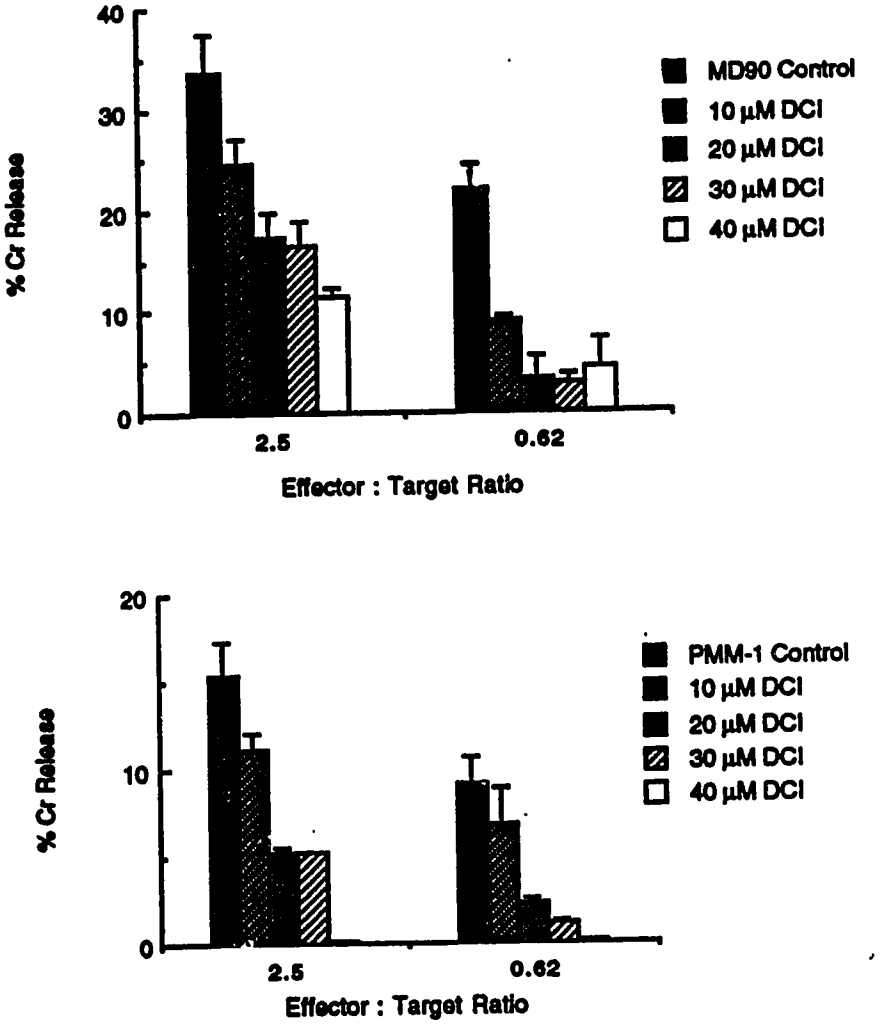


Figure 3.3: 3,4-dichloroisocoumarin inhibits PMM-1 and MD90 cytolysis. Antigen-activated PMM-1 and MD90 cells were incubated with EL4 targets for 6 hours with the indicated concentrations of DCI. DCI, dissolved in DMSO, was diluted in RHFM immediately prior to addition as a 10μl aliquot into a final volume of 200μl. Each point is the mean ± SD of triplicate determinations.

proteins interacted with the labelled substrate (Figure 3.4). The most interesting species was a band of approximately 16kDa with no significant activity in EL4 targets. Low levels were detected in PMM-1 cells, but not in MD90. The binding activity of this protein increased significantly during the course of the lytic assay while the activity of other proteins remained unchanged. These two results suggest the involvement of a serine proteinase in the mechanism of lysis employed by these cytolytic hybridomas. The presence of low levels of ^{3}H-DFP binding activity detected in PMM-1 cells prior to incubation with EL4 targets suggests that the activity may be localized to the effector cell. However, no conclusions are possible at this time.

Cytolysis involves poly-ADP ribosylation

Poly(ADP-ribose)synthetase is a nuclear enzyme which transfers the ADP-ribose moiety of NAD^{+} to an acceptor protein. Repetition of this process results in poly(ADP-ribosyl)ation of the protein. This enzyme has an absolute requirement for DNA with strand termini and is activated in response to DNA fragmentation. Critical depletion of NAD^{+} may be involved in cell death following synthetase activation (Yamamoto *et al.*, 1981).

The role of poly(ADP-ribose)synthetase in the cytolytic activity of the hybridomas was assessed utilizing two inhibitors: 4-amino-1,8-naphthalimide (ANA), a potent inhibitor of the synthetase (Banasik *et al.*, 1992) and nicotinamide, a weaker inhibitor, but also a scavenger of oxygen free radicals (LeDoux *et al.*, 1988; Banasik *et al.*, 1992). Results of these experiments are shown in Figure 3.5. The cytolytic activity of both PMM-1 and MD90 was significantly inhibited by ANA. The inhibition by nicotinamide was variable. It had little effect at either concentration on MD-90 mediated lysis. The activity of PMM-1 was inhibited significantly at higher effector to target ratios. In both cases equivalent concentrations of ANA were considerably more effective at preventing lysis than nicotinamide (i.e., complete inhibition of killing with 1mM ANA, but only 25% inhibition with 1mM nicotinamide

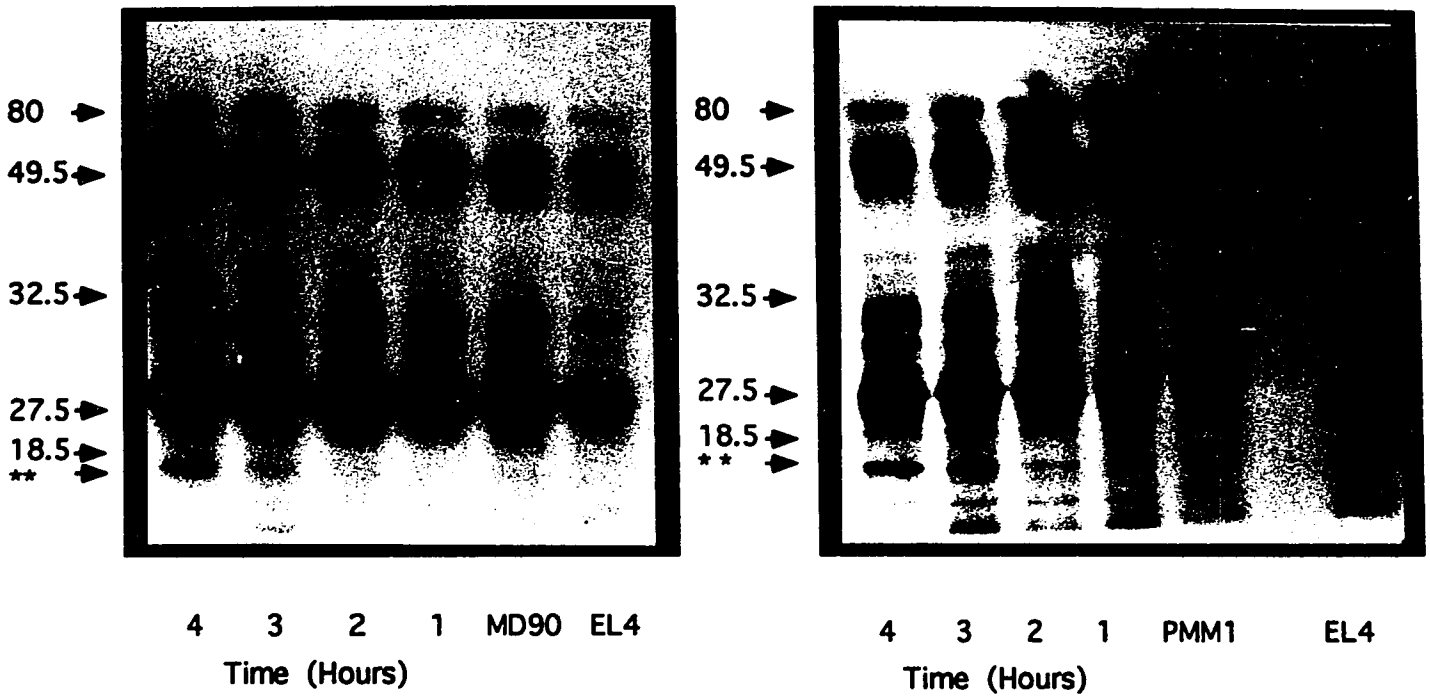


Figure 3.4: Induction of a 16kDa ^{3}H-DFP binding protein during cytolysis.

MD90 or PMM-1 effectors were incubated with an equal number of EL4 targets in RHFM for the times indicated (hours). Cells were harvested, washed in PBS, and lysed. Equivalent volumes of lysate were incubated with the ^{3}H-DFP, particulate matter was removed by centrifugation, and equivalent volumes of each sample were diluted in Laemmli sample buffer containing 0.1mM dithiothreitol. Labelled proteins were separated by SDS-PAGE (by Monica Varga) and detected by autoradiography. Arrows with numbers represent protein size (kDa). ** indicates induced activity.

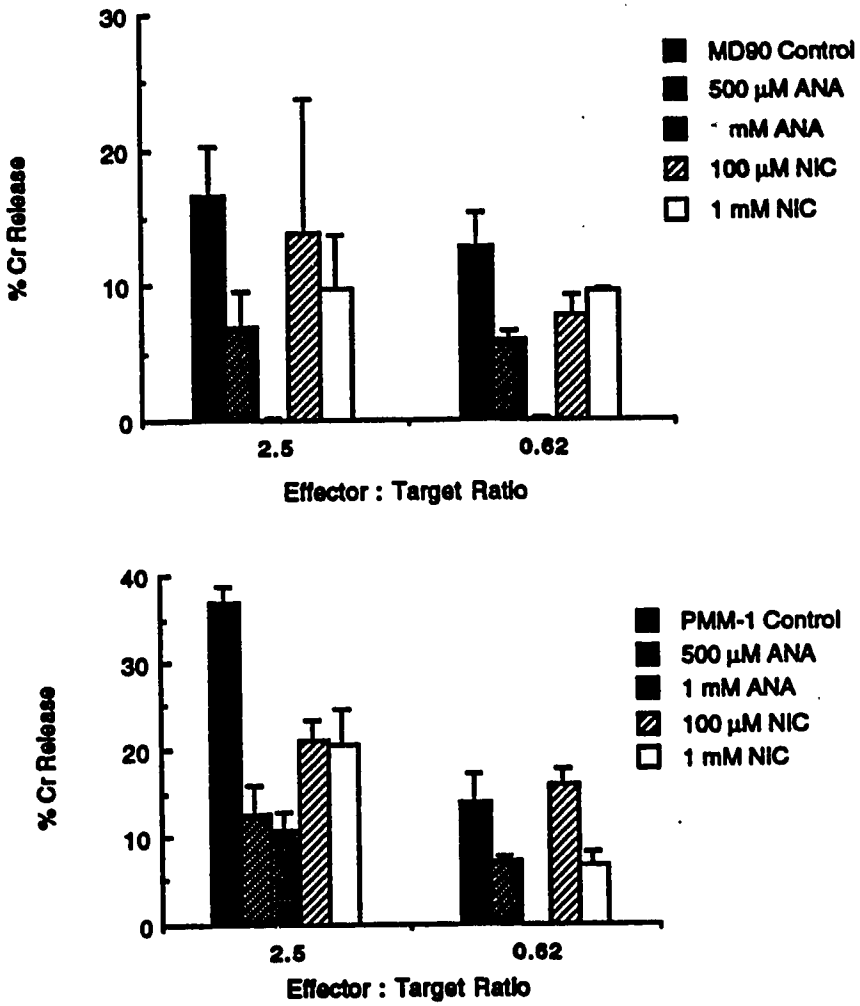


Figure 3.5: Poly(ADP-ribosyl)ation is involved in lysis mediated by PMM-1 and MD90.

Antigen-stimulated MD90 and PMM-1 cells were incubated with EL4 targets for 6 hours in the presence or absence of inhibitors. Dilutions of ANA and nicotinamide were made in RHFM immediately prior to addition, as a 10μl aliquot, to the assay wells. Results are the mean ± SD of triplicate determinations.

using the MD90 effectors). In contrast, neither the induction of DNA fragmentation nor the cytolytic activity of αCD3 mAb-activated mouse splenocytes was affected by either compound (Figure 3.6). These observations suggest that poly(ADP-ribosyl)ation is involved in hybridoma-mediated cytolysis.

TNF is not responsible for target cell lysis

Poly(ADP-ribosyl)ation is involved in TNFα-mediated cytolysis (Agrawal *et al.*, 1988). The observations with ANA suggested that TNFα may be the lytic mediator used by the hybridomas. This possibility was evaluated using several approaches.

Biological assays were carried out to determine if the hybridomas produced active TNF. The assay utilized detects both TNFα and LT at levels of less than 1 pg/ml (Branch *et al.*, 1991). Hybridomas were incubated for 18-22 hours in the presence or absence of antigen. In addition, aliquots of the EL4 cells, irradiated or not, were incubated for a similar amount of time. Three fractions from these cultures were examined: culture supernatants, cell lysates, and the membrane component. Table 3.2 summarizes the results. Low levels of TNF were detected in both the culture supernatants and membrane fractions of unstimulated cells. Antigen stimulation had no significant effect on the total amount of TNF activity detected. This observation argues against the involvement of TNF in hybridoma-mediated lysis.

The EL4 target cells produced significant quantities of TNF. Total levels were 0.18 pg/ml in untreated EL4 cells and 0.33 pg/ml following irradiation. It was therefore expected that they would not be sensitive to the levels of TNF produced by the activated hybridoma cells. This possibility was examined in a chromium release assay utilizing various concentrations of rMuTNFα (Table 3.3). The concentration of TNF required to achieve detectable lysis of the EL4 targets was more than 10,000x the levels of TNF found in the biological assays. Furthermore, even at these elevated concentrations, the extent of TNF-mediated lysis did not approach that achieved by the CTL

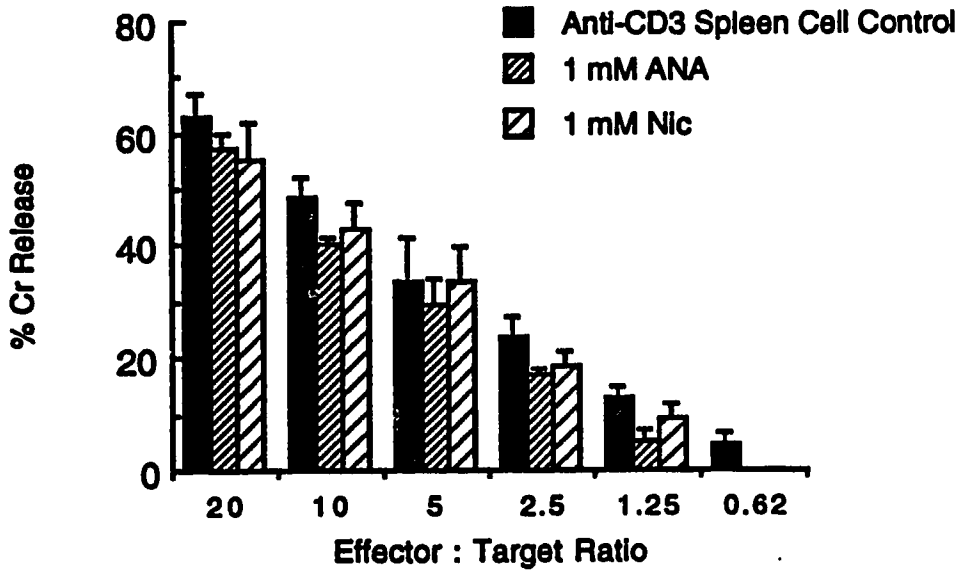


Figure 3.6: Poly(ADP-ribosyl)ation is not involved in the cytolytic activity of αCD3 stimulated spleen cells.

Mouse splenocytes were activated by incubation with a 1:500 dilution of αCD3 mAb for two days. Cytolytic activity was measured in the presence or absence of inhibitors in a four hour assay. Inhibitor dilutions were done as described in the legend to Figure 3.5. Results are the mean ± SD of triplicate determinations.

Table 3.2: TNF production by MD90 and PMM-1 cytolytic hybridomas.

	MD90		PMM-1	
	Unstimulated	Stimulated	Unstimulated	Stimulated
Supernatant	0.23 ± 0.023 (0.16)	0.28 ± 0.01 (0.08)	0.28 ± 0.00 (0.07)	0.29 ± 0.00 (0.07)
Cytosol	0.23 ± 0 (0.09)	0.25 ± 0.00 (0.11)	0.38 ± 0.01 (0.04)	0.25 ± 0.02 (0.12)
Membrane	0.24 ± 0.01 (0.13)	0.22 ± 0.00 (0.18)	0.21 ± 0.01 (0.20)	0.25 ± 0.00 (0.11)
Total TNF	0.38	0.37	0.31	0.30

Three fractions were collected from antigen-stimulated or unstimulated cultures of MD90 or PMM-1 cells. TNF activity in the various fractions was determined by Peter Lapchak using L929-8 fibroblast cells as described previously (Lapchak *et al.*, 1992). Results are expressed as the OD_{450} ± SD of triplicate determinations using a 1:2 dilution of each fraction. Numbers in brackets represent the TNF activity, in pg/ml, as determined from a rMuTNFα standard curve prepared at the same time. Total TNF activity is the sum of the TNF activity in all three fractions (pg/ml).

Table 3.3: The effect of rMuTNFα on EL4 target cells.

rMuTNFα (ng/ml)	% Specific ^{51}Cr Release (±SD)
1250	11.9 ± 6.7
625	14.9 ± 3.8
313	11.5 ± 4.6
156	10.6 ± 4.6
78	9.1 ± 3.6

rMuTNFα was diluted in a 100 μl volume of RHFM by doubling dilution. ^{51}Cr-labelled EL4 targets were added in a volume of 100 μl and the assay was incubated for five hours. Results are the mean ± SD of triplicate determinations. For conversion, 1 ng of TNFα is equivalent to 12 units.

hybridoma effectors in the same period of time (i.e., 14% maximum compared with 25 - 70%).

Initial characterization of these CTL hybridomas revealed that target cell lysis was antigen specific (Kaufmann *et al.*, 1981). This observation is difficult to reconcile with a mechanism of lysis mediated by a soluble lytic molecule. However, to test this possibility, cytotoxicity assays were carried out in which either the TNF-sensitive fibroblast cells, L929-8, or the inappropriate mouse target P815 were labelled with ^{51}Cr and added along with unlabelled EL4 targets in a standard 5 hour assay. Results are presented in Figure 3.7. Significant killing of EL4, the appropriate target, was observed while no direct lysis of either P815 or the TNF-sensitive L929-8 cells occurred, even in the presence of ConA to facilitate adhesion. In addition, there was no significant lysis of bystander L929-8 or P815 cells. These results argue against the involvement of a soluble lytic agent in the mechanism of lysis utilized by these hybridomas.

DNA Fragmentation is not a Cause of Lysis

Target cell DNA fragmentation, preceding chromium release, is induced by CTL and NK, but not by purified perforin (Duke *et al.*, 1983; 1989). The time course of DNA fragmentation and chromium release induced by the cytolytic hybridomas was examined (Table 3.4). Low levels of DNA fragmentation were observed. However, fragmentation did not precede target cell lysis. This degradation therefore occurs by a mechanism which varies from that utilized by CTL containing perforin and the CCPs. Further evidence for this suggestion is provided by the observation that the endonuclease inhibitor aurintricarboxylic acid (ATA) had no significant effect on the extent of either DNA fragmentation or target cell lysis (Figure 3.8). It was previously demonstrated that ATA inhibits DNA fragmentation, as well as target cell lysis, in a number of circumstances (Shi *et al.*, 1990; McConkey *et al.*, 1990; Chapter 5, this thesis).

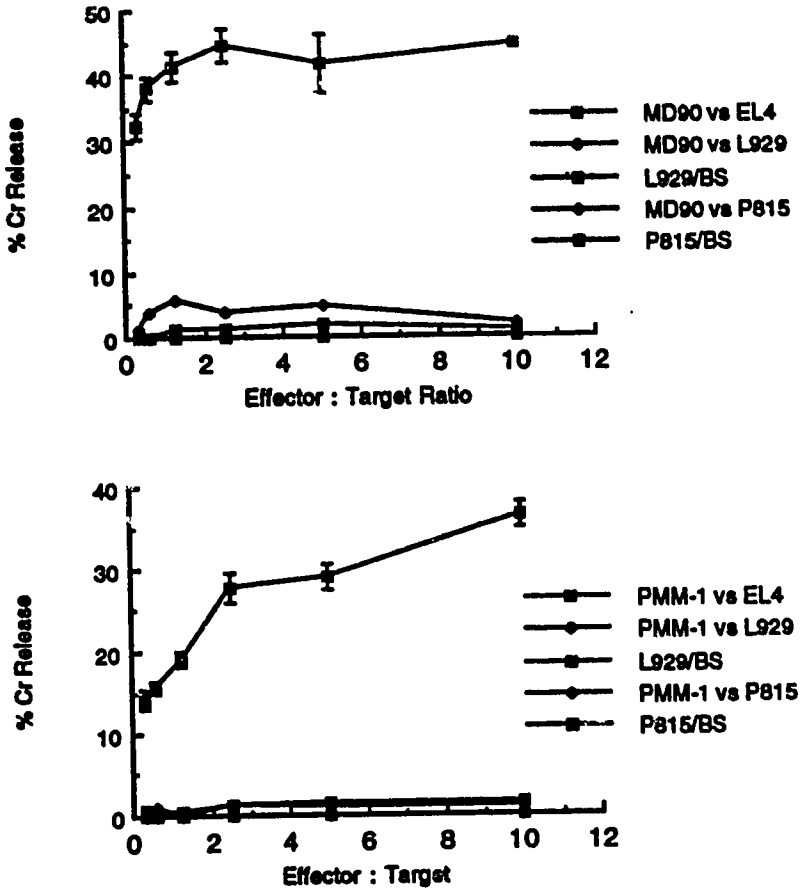


Figure 3.7: Cytolysis of EL4, P815, and L929-8 target cells.
Various numbers of MD90 or PMM-1 effectors were incubated with a constant number ($1x10^4$) of ^{51}Cr labelled EL4, P815, or L929-8 target cells for five hours in the presence of 2 μg/ml ConA. To determine bystander (BS) lysis of P815 or L929-8 cells, various numbers of MD90 or PMM-1 effectors were incubated as above with a mixture of unlabelled EL4 cells and labelled P815 or L929-8 cells (1:1 ratio of EL4:P815 or L929). Each point of the graph represents the mean ± SD of triplicate determinations.

Table 3.4: DNA fragmentation coincides with target cell lysis.

Time (Hours)	MD90		PMM-1	
	% ^{125}IUdR	% ^{51}Cr	% ^{125}IUdR	% ^{51}Cr
1	2.2 ± 0.9	N.D.	0.7 ± 0.1	N.D.
2	2.7 ± 0.6	0	3.6 ± 0.1	4.5 ± 0.6
3	3.3 ± 0.3	5.0 ± 0.2	5.0 ± 0.1	10.5 ± 0.8
4	4.6 ± 0.4	12.5 ± 1.6	6.0 ± 0.9	21.0 ± 0.3
5	N.D.	23.6 ± 3.3	N.D.	27.4 ± 4.5

N.D.: not determined.

Antigen-stimulated MD90 or PMM-1 cells were incubated with EL4 targets for the times indicated. Specific release was determined as described in Chapter 2. Results are the mean ± SD of duplicate samples for DNA fragmentation (^{125}IUdR) and triplicate samples for chromium release (^{51}Cr) at an E:T ratio of 5:1.

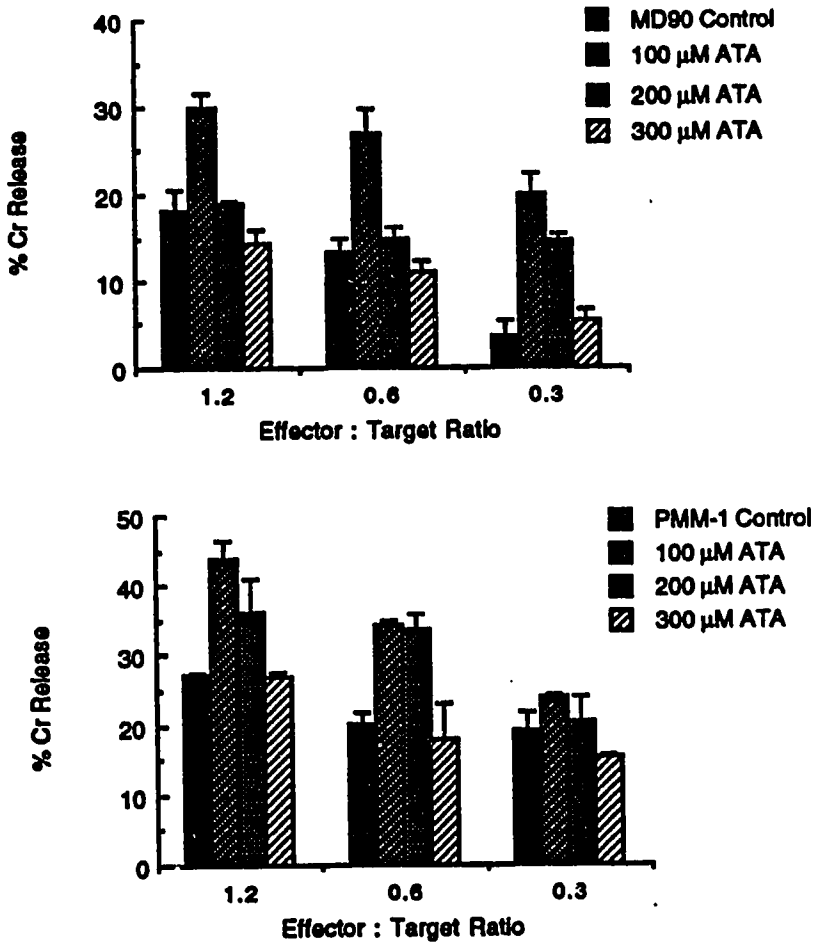


Figure 3.8: ATA has no effect on lysis mediated by MD90 or PMM-1. MD90 or PMM-1 hybridoma cells, activated with antigen, were incubated in a standard chromium release assay for 5 hours. Various concentrations of ATA, diluted in RHFM, were added in a 10 µl volume to assay wells at the start of incubation. Results are the mean ± SD of triplicate determinations.

DISCUSSION

The experiments described in this chapter were designed to characterize the mechanism of lysis used by various cytolytic hybridomas. These hybridomas resemble the CTL from which they were derived; they express the phenotypic markers of CTL, they lyse cells in an antigen-specific manner, and the kinetics of killing are similar to, although somewhat slower than, those observed with fresh PEL or MLC cells (Kaufmann *et al.*, 1981). Furthermore, lysis is inhibited by EDTA and cytochalasin B, agents which interfere with PEL and CTL mediated killing.

The PCR results confirmed the observation that neither PEL nor MLC hybridomas express detectable levels of perforin or CCP mRNA (Kaufmann and Berke, 1983; Berke, 1991). Although this study did not resolve the controversy in primary PEL surrounding the absence of perforin or proteinase activity in the presence of mRNA for these molecules, it did demonstrate that significant cytolytic activity can occur in the absence of perforin and CCP mRNA in a homogeneous CTL population. This observation argues against a unified lytic hypothesis based solely upon the action of cytolytic granules and their components.

The first insight into the potential mechanism of killing utilized by these hybridomas came from the observation that DCI caused a dose-dependent inhibition of cytolysis. This suggested the involvement of a serine proteinase. Neither the nature of this proteinase nor its potential role in cell death could be determined from these experiments. However, the finding that DCI did not completely inhibit the activity of either MD90 or PMM-1 suggests that the proteinase is not the sole mediator of cytolysis. Confirmation of this hypothesis requires isolation of the proteinase and determination of its sensitivity to DCI.

DFP is an irreversible substrate for serine proteinases (Cohen *et al.*, 1967), although it may nonspecifically interact with lipases. Time course studies of ^{3}H-DFP binding suggested that a small protein, approximately 16kDa, was induced during the process of cell lysis. This protein was not detectable in either EL4 or CTL alone. However, during cytolysis the activity increased. This result, taken together with the

proteinase inhibitor results, suggests that activation of a serine proteinase is involved in target cell lysis.

Protein synthesis is not required for target cell lysis by $CD8^+$ CTL (Golstein *et al.*, 1991). Presumably, the molecules required for killing are presynthesized and stored within cytoplasmic granules. Exocytosis of granule contents brings the mediators into direct contact with the target, thus circumventing the need for protein synthesis. In contrast, the requirement for protein synthesis during the lytic cycle was absolute for MD90 and almost complete for PMM-1 suggesting that at least some of the molecules involved in cytolysis were generated following specific contact between effector and target. This is analogous to the situation with $CD4^+$ cytolytic cells (Takayama *et al.*, 1991; Ozdemirli *et al.*, 1992). It is possible that one of the newly synthesized proteins is the 16kDa ^{3}H-DFP binding activity. Alternatively, the newly synthesized protein(s) may modulate the activity of this serine proteinase.

It is not possible to determine from these experiments whether protein synthesis and serine proteinase activation takes place within the effector or target or both. Contact between the two cells may induce these activities within the hybridoma to permit effective target cell lysis. A more likely explanation is that delivery of the lethal hit by the hybridoma triggers a number of processes within the target cell. They may be part of a repair mechanism or may function to enhance the signal generated by the cytolytic hybridoma. Further investigation is required to differentiate amongst these possibilities.

Although the biological function of poly(ADP-ribose) synthetase has not been established, its activity has been implicated in a variety of cellular processes including DNA repair, cell differentiation, control of the cell cycle, transcription, and alteration of the chromatin architecture (Boulikas, 1991). In addition, it plays a role in TNF-mediated lysis (Agrawal *et al.*, 1988; Vassali, 1992). The activity of this nuclear protein was induced in response to the DNA damage occurring during the process of target cell lysis. Only low levels of DNA fragmentation were observed concomitant with, and not

preceding, target cell lysis. The DNA damage measured in these assays corresponds to frequent double strand breaks which generate small fragments. It is possible that the hybridomas produce significant levels of undetectable (using this assay) single strand breaks which stimulate the activity of poly(ADP-ribose)synthetase. This suggestion corresponds with the observation that the endonuclease inhibitor ATA, shown to inhibit apoptosis (Shi *et al.*, 1990), as well as the lysis mediated by both NK (McConkey *et al.*, 1990) and CTL (Chapter 5), had no effect on lysis.

The role of poly(ADP-ribosyl)ation in hybridoma-mediated cell death was investigated utilizing two inhibitors, ANA and nicotinamide. ANA caused a dose-dependent inhibition of the lysis induced by both hybridomas. Inhibition by nicotinamide was variable. Although both effectively inhibit the activity of poly(ADP-ribose)synthetase (Banasik *et al.*, 1992), ANA is more effective. It was recently shown that very high levels (i.e., 40x more than those used in these experiments) of nicotinamide could interfere with the transmembrane signalling in CTL, thus preventing delivery of the lethal hit (Nowicki *et al.*, 1991). The variable effects observed with nicotinamide in these experiments suggest that this was not a problem.

Although poly(ADP-ribosyl)synthetase is likely to be activated in the target cell in response to DNA damage, it is possible that activation also occurs in the hybridoma effectors. Modification may increase the activity of certain critical proteins responsible, at least in part, for the lysis of the target cell. Thus, ANA may prevent activation of a key protein by inhibiting the synthetase. Alternatively, though not as likely in a short assay period, over-activity of poly(ADP-ribose)synthetase could alter cellular metabolism by inducing a critical depletion of NAD^+ stores. This possibility was suggested by the observation that CTL-mediated lysis was inhibited by nicotinamide, but DNA fragmentation was unaffected (Redegeld *et al.*, 1992).

Experiments utilizing labelled bystander cells (both the TNF-sensitive fibroblast cell line L929-8 and P815 tumor cells) argue

against a soluble mediator of lysis. If such a mechanism was employed by these hybridomas, significant lysis should have been observed when these cells were added along with an equal number of unlabelled EL4 targets. This was not the case. A specific interaction must be required between these effectors and their targets, since no direct lysis of either inappropriate target was observed even when contact was facilitated by the presence of the lectin ConA during the lytic assay.

The TNF family, TNF∝ and lymphotoxin (LT; TNFβ), has been implicated in the cytolytic activity of a number of different cell types including lymphocytes (Vassali, 1992). In general, TNF-mediated lysis occurs slowly over a period of 18-24 hours. However, a directed release of this soluble mediator or a membrane-bound form of TNF (Kriegler *et al.*, 1988) could potentially induce lysis in a much shorter period of time. This possibility was examined using a number of different approaches. Biological assays revealed low levels of TNF activity (TNF∝, LT, or a combination) associated with the hybridomas, but no correlation was seen between cytotoxicity and TNF activity. Furthermore, rMuTNF∝ at concentrations more than 10,000x in excess of those detected were unable to induce significant chromium release from the EL4 targets. Taken together, these observations argue against a role for TNF, either soluble or membrane-bound, in hybridoma-mediated cytolysis.

The mechanism of lysis utilized by the cytolytic hybridomas appears to consist of a complex series of metabolic events within the target cell. Initial damage involves nicking of the cellular DNA, possibly only in a single strand. This type of damage, though not readily detectable in the fragmentation assay, is sufficient to induce poly(ADP-ribosyl)ation of numerous proteins. Although the nature of the modified proteins was not determined, this step is essential in target cell lysis. Protein synthesis, absolutely necessary for MD90-mediated lysis and also important in the lysis mediated by PMM-1, may be induced in response to, or in concert with, the increased activity of poly(ADP-ribose) synthetase. It appears that a serine proteinase, potentially the one represented by the increased 16kDa ^{3}H-DFP binding activity, is

also involved in target cell lysis. This protein may be newly synthesized or simply activated in response to poly(ADP-ribosyl)ation. Regardless, the net result of these various changes is lysis of the target cell.

Experiments are now required to determine the nature of the ADP-ribosylated proteins and the induced serine proteinase. The requirement for protein synthesis during lysis suggests that the mediator(s) are expressed following contact between effector and target. Identification and isolation of these newly expressed proteins would therefore be useful in elucidating the steps involved in target cell lysis. A model of hybridoma-mediated lysis, as well as potential approaches to address unanswered questions, is presented in Chapter 7.

CHAPTER 4: DEVELOPMENT AND CHARACTERIZATION OF THE "PROGRAMMING FOR LYSIS" ASSAY.

INTRODUCTION

The process by which CTL contact and destroy appropriate target cells can be divided into a number of discrete phases which comprise the lytic cycle (Martz, 1975). The initial step involves a nonspecific interaction between the CTL and its potential target. This interaction is mediated by the adhesion molecules LFA-1 and CD2 on the T cell and ICAM-1 and LFA-3 on the target cell (Springer *et al.*, 1987). This tentative contact is fortified by specific recognition of a foreign peptide antigen presented in association with major histocompatibility (MHC) class I molecules on the target cell by the T cell receptor (TcR) of the CTL. Once formation of specific conjugates is complete, signal transduction within the CTL initiates the second phase of the lytic process.

Stimulation of the TCR, along with the associated CD3 complex, results in the transduction of two separate messages. One involves the generation of inositol 1,4,5-trisphosphate and diacylglycerol from phosphatidyl 1,4-bisphosphate (Gardner,1989). The second consists of increased intracellular calcium levels resulting from influx and release from intracellular stores (Gray *et al.*, 1987). Coupled with these biochemical processes are morphological changes required for delivery of the lethal hit. Within seconds of engaging the target cell, the microtubule organizing center and Golgi apparatus of the CTL migrate to the point of contact between the two cells (Kupfer and Dennert, 1984; Kupfer et. al., 1985). This movement is associated with condensation of the cytolytic granules near the effector-target junction (Yanelli *et al.*, 1986). The granule membrane fuses with the cellular membrane to release the contents of the granules into the synapse between effector and target. This stage is called delivery of the lethal hit or programming for lysis.

The final step in the lytic cycle involves detachment of the CTL from the target. Cell death does not require the continued presence of

the CTL. This final stage is therefore known as killer cell independent lysis (KCIL). The CTL having delivered the "kiss of death" is free to initiate another lytic cycle with a new target cell.

These steps have been delineated by using various conditions for incubation of effector and target (Martz, 1975; Golstein and Smith, 1976; Gately and Martz, 1977; Hiserodt *et al.*, 1982a). The first step involves incubation of the cells at a permissible temperature (i.e., 37°C) in the presence of $MgCl_2$ and EGTA. Conjugation between effector and target proceeds in the absence of calcium, but delivery of the lethal hit is not possible. The second phase of the cycle (programming for lysis) is initiated by addition of $CaCl_2$ which permits transmission of the death signal to the target cell. Termination of this stage is achieved using EDTA to chelate magnesium and calcium, thus preventing any further conjugate formation or delivery of the lytic message. In some instances this KCIL phase is maintained by the addition of high molecular weight dextran which prevents reassociation without producing colloid osmotic damage to the target cell (Martz, 1975).

This type of assay system has been utilized to examine the sensitivity of each stage to a variety of drugs (Gately *et al.*, 1980; Hiserodt *et al.*, 1982b; Brogan and Targan, 1986). Comparison between the effect of an inhibitor present for the entire lytic assay and only for a specific phase of the assay indicates whether there is a preferential inhibition of a certain stage, or if all steps of the cycle are equally sensitive to the agent.

One potential drawback to the use of such a system is that the various chelators, and possibly dextran, have the potential to interfere with the processes modulated by the drug of interest. For example, attempts to study the effects of a reagent on a calcium-dependent enzyme in the presence of EDTA may make interpretation of the results difficult. That is, it would not be possible to determine if inhibition was due to chelation of calcium by EDTA or if it resulted from a specific effect by the compound added. Assessment of target cell fate is impossible following the use of EDTA, with or without dextran, for two reasons. First, it is difficult to effectively separate the CTL

from the targets, and secondly, the presence of EDTA and dextran may interfere with long-term (i.e., more than 4-6 hours) target cell viability.

This chapter summarizes the development and characterization of a novel system for programming target cells for lysis. The method relies on the use of a newly-isolated adherent subclone of an antigen-dependent cloned CTL line (Havele *et al.*, 1986). Target cells are easily separated from the effectors, thus permitting determination of their fate over longer periods of time. Included in the discussion are the potential uses, as well as the limitations, of this assay.

RESULTS

Adherent CTL induce DNA fragmentation and chromium release in target cells

Conventional cytolytic assays do not lend themselves to evaluation of target cell viability 24 - 48 hours after initiation of the lytic cycle. In order to examine long-term target cell fate it was necessary to devise a method of separating them from CTL. An adherent population of the CTL clone 21.9(I), designated CTL 21.9(IA), was isolated. Its ability to lyse target cells, without loss of adherence, was examined.

Initial observations suggested that incubation of the adherent cells with a 1:500 dilution of αCD3 mAb (Leo *et al.*, 1987) for 18 - 24 hours had two effects. First, proliferation of the cells was not observed during the period of incubation with the antibody. Secondly, cytolytic activity was greatly enhanced, regardless of whether the CTL were exposed to targets while still attached to the culture dish or while in solution. CTL 21.9(IA) cells were therefore routinely plated at the desired concentration and stimulated overnight with αCD3 mAb prior to addition of targets.

The programming for lysis assay consisted of incubating appropriately labelled target cells with various numbers of adherent CTL for 5 to 60 minutes. Target cells were removed carefully to avoid disruption of the adherent CTL monolayer, and target cell fate was followed using both chromium release and DNA fragmentation assays.

Results presented in Table 4.1 indicate that coincubation of CTL and target cells for 20 - 40 minutes was sufficient to induce both DNA fragmentation and chromium release from the target cell during the subsequent KCIL phase. One consistent observation was that shorter exposure times (i.e., less than 20 minutes) induced significant levels of DNA fragmentation with little corresponding chromium release. Although lower effector to target ratios were utilized (not shown), the levels of chromium release were too low to be of significance. In further experiments, programming times of 15 - 60 minutes were routinely used.

Target cell viability parallels DNA fragmentation

Establishment of a method for assessing extended target cell viability following CTL contact was one objective of this study. This was assessed by programming targets for lysis and then incubating them for 24 hours under normal growth conditions. Target cell viability, compared to similarly manipulated control cells, decreased as the period of exposure to CTL increased (Table 4.2). Interestingly, viability closely correlated with the severity of DNA fragmentation detected at 90 minutes, but not with levels of chromium release at this time. Table 4.2 indicates that even limited exposure to CTL interferes with long-term target cell survival. The significance of these observations was twofold. First, it was possible to examine the KCIL phase over extended periods of time in the absence of CTL and without the addition of EDTA and/or dextran. Secondly, DNA fragmentation was a better indicator than chromium release of CTL-induced target cell death.

Lysis involves CTL degranulation

Several mechanisms have been proposed to explain CTL-mediated target cell lysis (Henkart, 1985; Young and Cohn, 1986; Clark et al., 1988; Tschopp and Jongeneel, 1988). One of these involves the directed release of cytolytic granules from the CTL into the confined junction between the effector and target. Exocytosis is assessed by measuring the level of granzyme A (BLT esterase; BLTE) activity in the culture

Table 4.1: Programming for lysis results in target cell rupture (^{51}Cr release) and DNA fragmentation ($^{125}IUdR$ release).

Time of programming (min)	% specific ^{51}Cr release	% specific $^{125}IUdR$ release
5	9.7	7.5
10	4.8	10.2
20	7.9	19.0
40	21.5	23.5
60	46.0	24.2
4 hour control	69.0	25.3

CTL 21.9(IA) cells were stimulated for 18 hours with a 1:500 dilution of αCD3 mAb. Appropriately labelled P815 target cells were incubated in the wells with CTL for various periods of time and were removed for assays as described in Chapter 2. Results are presented for a 10:1 effector to target ratio with a 4 hour incubation for ^{51}Cr release (triplicate determinations) and a 3 hour incubation for $^{125}IUdR$ release (duplicate determinations).

Table 4.2: Target cell viability correlates with the extent of DNA fragmentation.

Time	% ^{51}Cr Release		% ^{125}IUdR Release		% Viability	
(min)	A	B	A	B	A	B
15	2.7	2.1	14.7	12.1	74.3 ± 2.7	80.4 ± 4.4
30	7.2	5.1	52.1	46.3	46.0 ± 3.5	56.4 ± 3.0

P815 target cells were programmed for lysis by incubation at a 10:1 E:T ratio with CTL 21.9(IA) effectors for the times indicated (Time). Levels of fragmentation (^{125}IUdR) and chromium (^{51}Cr) release were determined at 1.5 hours. At the same time unlabelled targets treated similarly were distributed into flat-bottom 96-well plates and incubated under normal conditions for 24 hours. Target cell viability was assessed using the colorimetric MTT assay as described in Chapter 2.

supernatant in the presence and absence of stimuli (i.e., target cells) which induce degranulation (Takayama *et al.*, 1987).

The involvement of exocytosis in CTL 21.9(IA)-mediated lysis was examined. Release of BLTE activity was detected at the earliest time point assessed (i.e., 15 min.), although levels were not significant until 30 minutes (Table 4.3). More importantly, stimulated release increased with longer periods of co-incubation. This time course parallels the results observed with both cell lysis and DNA fragmentation. That is, longer exposure of targets to CTL induces higher levels of degranulation. There was a corresponding increase in target cell damage.

It was essential to determine if degranulation during the programming event contributed to target cell death. Cyclosporin A (CsA) is an immunosuppressive agent with numerous effects on the immune system (Sigal and Dumont, 1992). For example, CsA inhibits CTL effector functions by preventing granule exocytosis (Havele and Paetkau, 1988; Trenn *et al.*, 1989). The role of degranulation in the programming assay was determined by programming in the presence or absence of 200 ng/ml CsA. Results of two representative experiments are presented in Table 4.4. Although the levels of all parameters assessed varied from one experiment to the other, the trend was apparent in both instances: CsA inhibited degranulation and reduced both chromium release and DNA fragmentation in the target. These results implicate granule exocytosis in the lytic mechanism used by CTL 21.9(IA).

Target cell recovery from the assay is consistent

Previous analysis of the lytic cycle revealed that the conjugates formed between effectors and targets were very stable (Martz, 1975; Gately and Martz, 1977). Physical disruption, such as vortexing, was insufficient to separate the cells. Neither was EDTA alone adequate. The combination of physical force and chelation of magnesium was required.

One concern raised by the studies presented above was that few target cells would be recovered from the programming assay at the

Table 4.3: Release of BLTE occurs during programming for lysis.

Programming time (Min)	Spontaneous release	Stimulated release	Magnitude of increase
15	130 ± 7 (9.8)	148 ± 6 (11.2)	1.1x
30	142 ± 8 (10.7)	221 ± 19 (16.7)	1.6x
45	175 ± 22 (13.2)	318 ± 45 (24.0)	1.8x
60	181 ± 7 (13.6)	427 ± 24 (32.2)	2.4x

CTL 21.9(IA) effectors were incubated with P815 targets at a ratio of 10:1 for the times indicated. Cell-free supernatants were reacted with the colorimetric substrates for 3 hours, and the absorbance was determined at 410 nm. Results are presented as the specific OD readings. Numbers in brackets represent the percent of total release determined by detergent lysis of effector cells. Magnitude of increase refers to the ratio of specific to spontaneous release. The results are representative of 3 separate determinations.

Table 4.4: Inhibition of degranulation blocks lysis and DNA fragmentation.

Group	BLTE Release		% ^{51}Cr Release		% ^{125}IUdR Release	
	A	B	A	B	A	B
Control	187	198	6.4	18.0	10.7	20.9
0.2 μg/ml CsA	65	134	0	6.8	4.4	12.9

The results of two separate experiments are presented. P815 targets were programmed for lysis over a 40 minute period at an E:T of 10:1. BLTE results represent the activity in 100μl of supernatant following a 1.5 hour incubation (expressed as the OD_{410} minus background). Incubations for the chromium release assay were 5 and 6 hours (A and B) respectively, and 3 and 3.5 hours for the fragmentation assay.

various time points due to their tight association with the adherent effectors. Table 4.5 indicates that a consistent number of target cells was recovered from the assay wells at each of the time points. In addition, there was little variation in recovery from one incubation period to the next. No assumptions can be made regarding the percentage of recovered cells which have received the "kiss of death". However, as discussed later, the amount of DNA fragmentation detected may reflect the number of targets which have been lethally hit.

Further analysis indicated that there was no significant transfer of the CTL with the targets following programming for lysis. This was assessed in two ways. First, CTL 21.9(IA) were incubated with either P815 targets or RHFM for equivalent amounts of time. The supernatants were removed and the adherent CTL were harvested and counted. No significant differences in recovery were detected following incubation with or without targets. Secondly, the supernatants collected above were incubated with chromium-labelled P815 targets. No lysis was detected using either the P815 or RHFM fraction. These results indicate that a consistent number of programmed targets can be recovered without significant CTL contamination.

DISCUSSION

This chapter outlined the development of a system to program target cells for lysis and to subsequently follow their fate in the absence of effector cells. Unlike previous systems for examining the KCIL phase of target cell lysis (Martz, 1975; Golstein and Smith, 1976), this method did not require the addition of cation chelators or viscous solutions to induce and maintain the KCIL phase. Therefore, not only was it possible to examine target cell fate over a normal 3-4 hour period, but target cell viability could also be examined as much as 24-48 hours later.

Early studies on the phases of the lytic cycle suggested that the initial step, formation of effector - target conjugates, could be completed within 3 minutes at permissive temperatures (i.e., 37°C) in the presence of magnesium (Martz, 1975; Gately and Martz, 1977).

Table 4.5: Target cell recovery is consistent.

Experiment	15 Minutes	30 Minutes
1	47.5 ± 2.5	16.9 ± 1.7
2	33.6 ± 3.2	ND
3	46.3 ± 4.6	35.1 ± 4.5
4	28.7 ± 1.8	36.5 ± 5.1
Mean ± SD	39.0 ± 9.3	29.5 ± 10.9

ND = not determined

Values in the table represent the percentage of P815 cells, determined on the basis of ^{125}IUdR cpm, recovered following 15 or 30 minutes of programming with CTL 21.9(IA) effectors.

However, contact in these instances was facilitated by centrifugation of the assay plates. Delivery of the lethal hit, a temperature-sensitive, calcium-dependent phase, occurs during a period of 10-20 minutes following establishment of appropriate conjugates (Gately *et al.*, 1980; Hiserodt *et al.*, 1982a). The final phase, KCIL, takes place over several hours, even in the absence of effectors (Martz, 1975; Gately and Martz, 1977).

Although it was not possible to facilitate contact by centrifuging the cells, the results presented in Table 4.1 indicate that sufficient cell contact occurred within 20 minutes to induce high levels of DNA fragmentation, and detectable chromium release, during the subsequent KCIL phase. DNA fragmentation occurred earlier, and to a greater extent, than chromium release. Furthermore, only the levels of DNA fragmentation correlated with target cell viability over extended periods of time (Table 4.2). This point will be elaborated upon later.

The kinetic properties of CTL 21.9(IA)-mediated lysis resembled those of normal CTL or NK, but further characterization of the lytic mechanism utilized by these cells was required. Although release of BLTE activity does not parallel the amount of lysis observed (Ratner and Clark, 1991; Smyth *et al.*, 1992), the release of BLTE is indicative of degranulation. CTL 21.9(IA) effectors exhibited a time dependent release of BLTE activity suggesting that degranulation is involved in lysis.

Among its numerous effects on the immune system, CsA can inhibit degranulation of various cells including CTL (Havele and Paetkau, 1988; Trenn *et al.*, 1989). This results in partial inhibition of chromium release, although others have reported no effect on lysis (Lancki *et al.*, 1989; Smyth *et al.*, 1992). Complete inhibition of BLTE release was not observed in the programming assay, but CsA produced a corresponding decrease in the levels of both chromium release and DNA fragmentation. These results, in combination with the detection of BLTE activity in the supernatant, suggested that degranulation is involved in the mechanism of killing utilized by CTL 21.9(IA).

As mentioned previously, long-term target cell viability following delivery of the lethal hit was more closely related to the extent of DNA fragmentation than to the amount of chromium release detected. DNA fragmentation is not critical for the death of all types of targets or in all circumstances (Howell and Martz, 1987; Ucker *et al.*, 1992). However, target cells have the ability to repair damage induced by limited exposure to perforin (Jones *et al.*, 1990), while DNA fragmentation is irreversible (i.e., detection of 30% fragmentation indicates 100% fragmentation in 30% of the cells - Cohen *et al.*, 1992). The observation that limited exposure of targets to CTL (i.e., 20 minutes or less) induced significant levels of DNA fragmentation and target cell death, with little membrane damage indicates that this programming assay may be useful for studying DNA fragmentation without the complication of target cell rupture. Furthermore, it suggests that measurement of chromium release is not always an accurate means of assessing target cell damage. Rather, chromium release simply reflects the amount of exposure to perforin.

The assay described in this chapter provides a method for controlling the extent of contact between the effector and the target without addition of various inhibitors. It also permits control over the amount of perforin-induced damage. Furthermore, it facilitates examination of drug effects only on the KCIL stage of lysis. This newly developed programming for lysis assay has the potential to provide useful information on a number of parameters related to target cell death. More importantly, these evaluations can be made without further input from the CTL.

CHAPTER 5: THE ROLE OF DNA FRAGMENTATION IN CTL-MEDIATED LYSIS

INTRODUCTION

Cytotoxic T lymphocytes (CTL) are one of the key effectors in the cell-mediated defense against malignant and virally-infected cells. The mechanisms by which target cell destruction is accomplished have been extensively studied (for review see Henkart, 1985; Young, 1989; Tschopp and Nabholz, 1990). The most widely accepted model of target cell lysis involves the exocytosis of cytolytic granules from the CTL, the contents of which are capable of destroying the target cell. However, ample evidence exists for alternate mechanisms of inducing lysis in the absence of granule exocytosis (Berke and Rosen, 1987; Ostergaard *et al.*, 1987; Trenn *et al.*, 1987; Ostergaard and Clark, 1989). In general, two modes of target cell death have been observed following contact with a CTL.

Necrotic death can be induced by the calcium-dependent pore-forming protein perforin (cytolysin) (Podack and Konigsberg, 1984; Masson and Tschopp, 1985). This protein is a major constituent of the cytoplasmic granules in both CTL and natural killer (NK) cells. Morphological features of necrosis include dilation of the endoplasmic reticulum, an increase in mitochondrial volume, flocculation of the nuclear chromatin, and cell swelling (Wyllie *et al.*, 1980). These changes can be attributed to perturbations of the cytoplasmic membrane which disrupt the integrity of the permeability barrier. Polymerization of perforin and its insertion into the membranes of target cells results in the formation of distinct pores which allow small ions and solutes to pass through the membrane. The net result is that normal extracellular/intracellular gradients are destroyed and osmotic rupture of the target occurs. In this way, perforin-mediated lysis does not require active participation of the target in its own demise, i.e., inhibitors of protein or RNA synthesis are unable to protect the cell from its ultimate fate (Zychlinsky, 1991).

Necrotic death of a cell is not associated with degradation of the nuclear chromatin until the later stages. Furthermore, this degradation

is a random event mediated by proteinases and endonucleases which have gained access to the DNA due to the osmotic rupture of internal cellular membranes. However, CTL induce a controlled form of DNA degradation which precedes the loss of membrane integrity (Duke *et al.*, 1983; Russell, 1983). This type of DNA breakdown can be induced by isolated cytolytic granules (Podack *et al.*, 1988), although evidence to the contrary has been published (Gromkowski *et al.*, 1988; Duke *et al.*, 1989). Morphological studies of target cell death (Sanderson, 1976; Liepins *et al.*, 1977; Matter, 1979), as well as the early occurrence of DNA fragmentation into a nucleosomal ladder (Duke *et al.*, 1983; Russell, 1983; Gromkowski et al, 1986; Howell and Martz, 1987), suggest that CTL induce apoptosis in their cognate targets.

Apoptosis is characterized by chromatin condensation, cell surface blebbing, and fragmentation of the cellular DNA (Wyllie *et al.*, 1980). It has been observed in a wide variety of cell types and is induced by a range of conditions, often under circumstances in which the processes leading to cell death are regulated or "programmed". One example of relevance is the induction of apoptosis in immature thymocytes and some T cell hybridomas following activation via the T cell receptor. This activation-induced apoptosis is likely to be the mechanism of negative selection in which thymocytes are selectively removed from the maturation pathway upon interaction of their T cell receptors with specific ligand (Shi *et al.*, 1989; Smith *et al.*, 1989). Unlike necrosis, apoptosis requires active participation of the cell in its own death. It has been shown that macromolecular synthesis, in many instances, is required for apoptotic death (Cohen and Duke, 1984; Ucker *et al.*, 1989; Odaka, 1990; Shi *et al.*, 1990).

A possible relationship between CTL-mediated killing and apoptosis was suggested by studies with a glucocorticoid-resistant thymoma (Ucker, 1987). DNA fragmentation could not be induced in these cells by either glucocorticoids or by CTL. A single-step reversion of glucocorticoid resistance restored sensitivity to both. However, this result is controversial (Dennert *et al.*, 1988). The role of apoptosis in CTL-mediated death has been challenged by observations that addition of

inhibitors of DNA, RNA, or protein synthesis at the beginning of a cytotoxicity assay has no effect on CTL-induced cell lysis (Duke *et al.*, 1983). However, the contribution of perforin-induced osmotic lysis was not considered. In contrast, other studies showed that preincubation of the target cells with inhibitors of RNA or protein synthesis resulted in significant inhibition of both DNA fragmentation and cell lysis (Zychlinsky *et al.*, 1991). Several examples of apoptotic cell death occurring in the absence of protein synthesis have also been noted (Waring, 1990; Batistatou and Greene, 1991; Collins *et al.*, 1991). These results indicate that many questions remain to be resolved regarding the relationship between apoptosis and CTL-mediated killing. Furthermore, the role of DNA fragmentation in both types of cell death remains to be clarified.

Aurintricarboxylic acid is a potent inhibitor of endonuclease activity (Hallick *et al.*, 1977). ATA inhibits apoptosis and the associated DNA fragmentation in thymocytes and in a T cell hybridoma (McConkey *et al.*, 1989; Shi *et al.*, 1990). Several reports have appeared in the literature which suggest that CTL induce apoptosis in target cells (Russell, 1983; Zychlinsky, 1991; for review see Golstein *et al.*, 1991). However, the observation that purified perforin induces osmotic lysis in the absence of DNA fragmentation has challenged the significance of nucleosomal damage in target cell death (Tschopp and Nabholz, 1990).

The purpose of the experiments presented in this chapter was to determine the role of DNA fragmentation in target cell lysis induced by CTL. ATA was a potent inhibitor of CTL-mediated lysis and DNA fragmentation. At the doses required to block target cell death, there was no effect on cytotoxic granule exocytosis, suggesting that neither CTL-target interactions nor intracellular signal transduction were blocked. Studies by collaborators demonstrated inhibition of a Ca^{++} and Mg^{++}-dependent endonuclease activity in isolated nuclei and inhibition of lysis by purified perforin. The programming for lysis assay (described in Chapter 4) was used to demonstrate inhibition of DNA fragmentation by ATA when the effect of perforin was minimized by

limited exposure of the targets to CTL. This inhibition was directly related to a decrease in chromium release and increased viability of the targets one day later. These results provide evidence that target cell death is enhanced by DNA fragmentation.

RESULTS

ATA inhibits CTL-induced lysis and DNA fragmentation

Initial experiments examined the effect of ATA on the cytolytic activity of CTL in an *in vitro* cytotoxicity assay. The presence of micromolar concentrations of ATA effectively inhibited target cell lysis induced by either mitogen (ConA) stimulated (Figure 5.1) or alloantigen-stimulated (MLR) CTL (Figure 5.2). The degree of inhibition depended upon the concentration of ATA used. The cloned cytotoxic T cell line CTL 21.9(I) was considerably more sensitive to ATA (Figure 5.3).

Consistent with the cytotoxicity results, there was also a significant reduction in the magnitude of target cell DNA fragmentation induced by CTL 21.9(I) (Figure 5.4). Quantitative analysis of the ATA effect on DNA degradation is presented in Table 5.1 These results clearly demonstrate that ATA inhibits both target cell lysis and DNA fragmentation resulting from contact with the CTL.

The specificity of this effect was examined with respect to a number of parameters related to CTL function. The demonstration that ATA blocks the interaction of HIV I with the CD4 molecule on the surface of T lymphocytes (Schols *et al.*, 1989), suggested that ATA might prevent the conjugation between effector and target. Results presented in Table 5.2 suggest that ATA does not interfere with either effector-target interactions or signal transduction within the CTL. Antigen-specific BLT esterase release (Takayama *et al.*, 1987) from the CTL was unaffected by the presence of ATA. Neither did ATA have any effect on the activity of the BLT esterase itself.

The effect of ATA on the late phases of the lytic cycle, programming for lysis and killer cell independent lysis (KCIL), was examined by adding ATA after sufficient time had passed for the majority

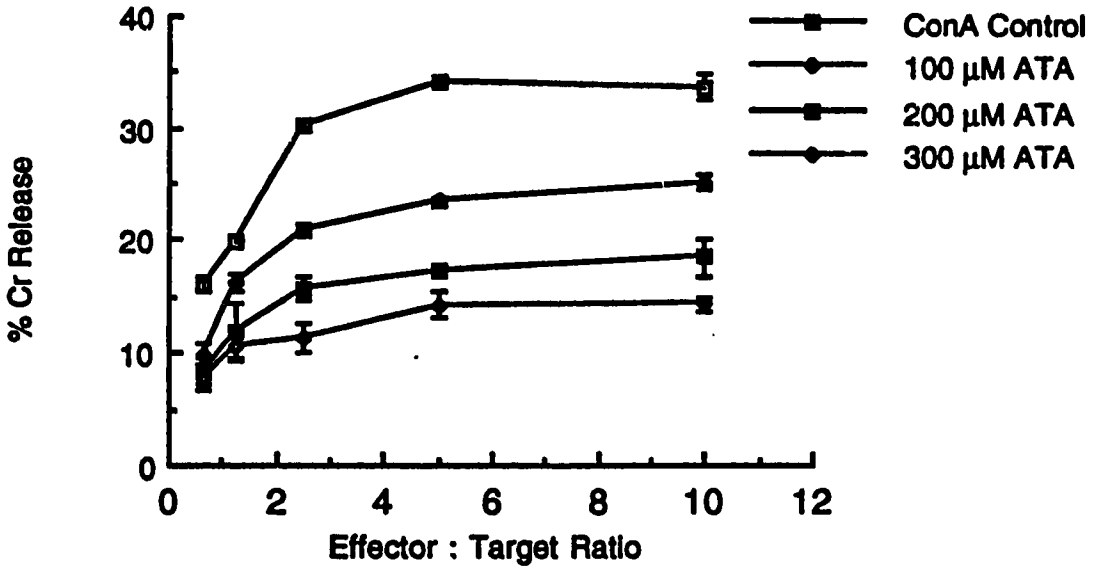


Figure 5.1: ATA inhibits lysis induced by mitogen-activated CTL. CTL, generated by stimulation of splenocytes with ConA, were incubated with chromium labelled P815 targets in the presence of various concentrations of ATA. Cytotoxicity was measured as described in Chapter 2. Each point is the mean ± SD of triplicate determinations.

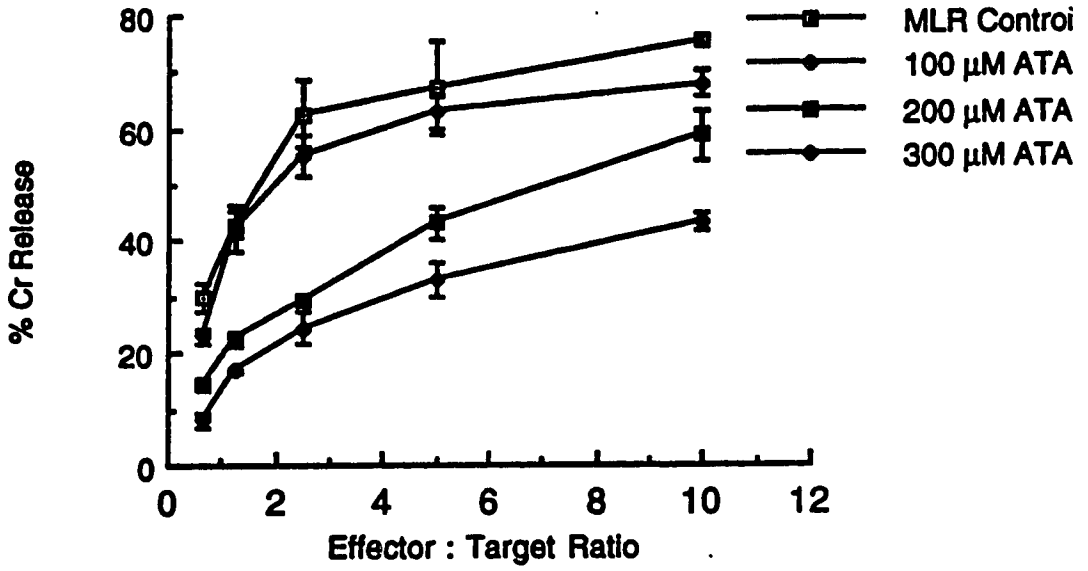


Figure 5.2: Inhibition of target cell lysis induced by MLR CTL.
CTL generated in an MLR were incubated with P815 targets for four hours in the presence of ATA. Results are the mean ± SD of triplicate determinations.

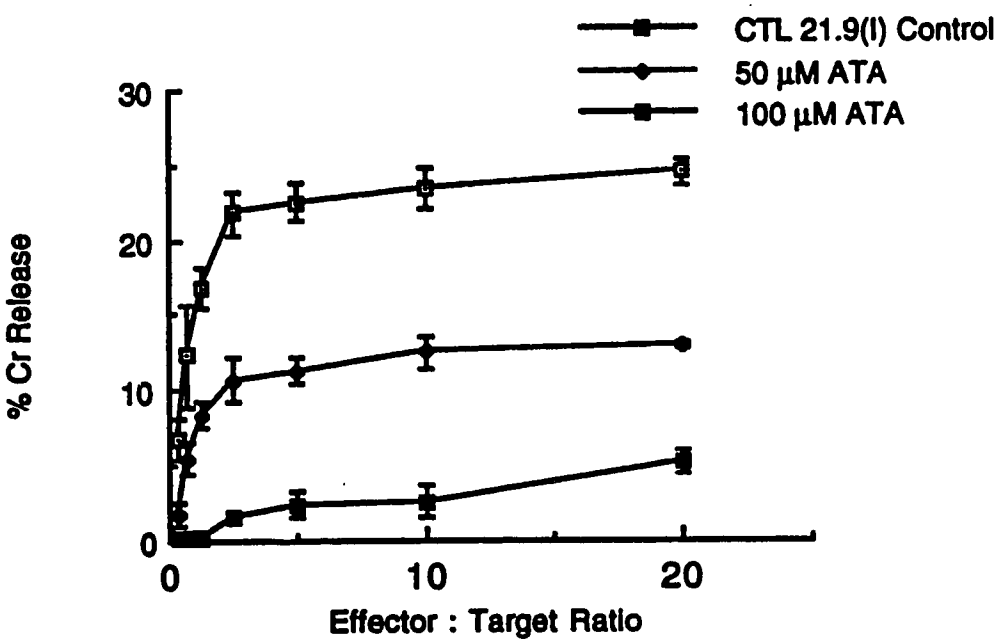


Figure 5.3: ATA inhibits cytolysis induced by the CTL clone CTL 21.9(I). CTL 21.9(I) effectors were incubated with P815 targets for four hours in the presence of various concentrations of ATA. Cytotoxicity was measured as described in Chapter 2. Results are the mean ± SD of triplicate determinations.

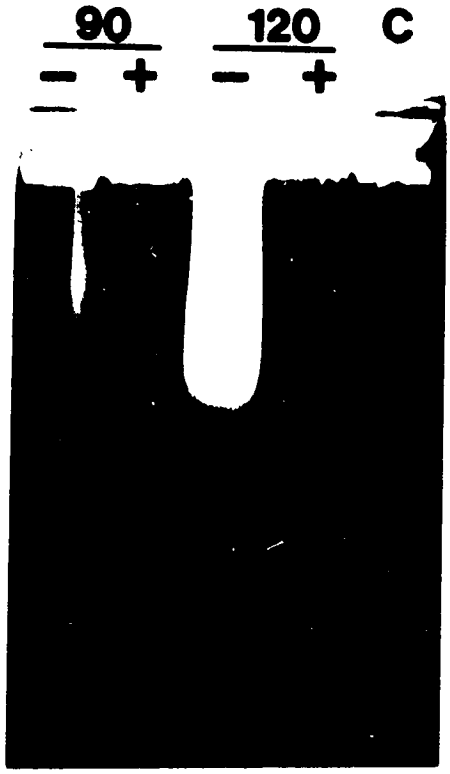


Figure 5.4: ATA inhibits CTL-mediated DNA fragmentation.
CTL 21.9(I) effectors were incubated for 90 or 120 minutes with P815 targets in the presence (+) or absence (-) of 200 μM ATA. Prior to lysis, total cellular DNA was isolated, electrophoresed in an agarose gel, and detected by UV following ethidium bromide staining. P815 targets, in the absence of effectors, were run as a control (lane C).

Table 5.1: ATA inhibits fragmentation induced by both MLR and CTL 21.9(I) effectors.

MLR		CTL 21.9(I)	
- ATA	+ ATA	- ATA	+ ATA
84.7 ± 2.4	53.0 ± 2.3	76.2 ± 1.0	24.8 ± 1.4

MLR or CTL 21.9(I) effectors were incubated for 3 hours with P815 targets at a 10:1 E:T ratio in the presence or absence of 200 μM ATA. Fragmentation was assessed as described in Chapter 2. Each point is the mean $\pm$ SD of triplicate determinations.

Table 5.2: ATA has no effect on contact-mediated effector functions.

Treatment	Basal BLTE Release	Stimulated BLTE Release
Control	8.3 ± 2.4	18.8 ± 1.2
100 μM ATA	8.9 ± 2.0	24.7 ± 2.1
200 μM ATA	10.3 ± 1.9	25.7 ± 3.1

MLR effectors (basal) or effectors plus targets (stimulated) were incubated together for four hours in the presence of various concentrations of ATA. A fraction of the supernatant was assayed for BLT esterase activity as outlined in Chapter 2. Results are expressed as the percent of total activity derived from a detergent lysate. Each number is the mean ± SD of triplicate determinations from three separate experiments.

of the targets to have been contacted by the CTL. Results presented in Figure 5.5 indicate that ATA prevented both fragmentation and lysis when added as late as 60 minutes after initiation of programming. Therefore, ATA inhibits KCIL, as well as programming for lysis.

ATA inhibits cytolysis induced by isolated granular proteins

My collaborators in this study (from the laboratory of Dr. A.H. Greenberg, University of Manitoba) examined the effect of ATA on two important components of the cytolytic granules: 1) perforin, which induces target cell lysis in the absence of DNA fragmentation (Tschopp and Nabholz, 1990) and 2) fragmentin 2, a rat NK granule proteinase homologous to murine cytotoxic cell proteinase 1 (CCP1; Granzyme B), which cooperates with perforin to induce DNA fragmentation and increase target cell lysis (Shi *et al.*, 1992b).

Purified perforin alone induced target cell lysis in the absence of DNA fragmentation (Table 5.3). Fragmentin alone, like other granular serine proteinases (Munger *et al.*, 1988; Hayes *et al.*, 1989), had no effect on either fragmentation or lysis. More importantly, chromium release increased significantly in the presence of perforin plus fragmentin. This increase could be attributed to the induction of DNA fragmentation by fragmentin. Although ATA inhibited perforin-mediated lysis in a dose-dependent manner, there was a marked reduction of both the chromium release and the DNA fragmentation which occurred in the presence of perforin plus fragmentin (i.e., 3 μM ATA inhibited both DNA fragmentation and chromium release by approximately 50% relative to control cells with no ATA).

The inhibition of lysis mediated by purified perforin using ATA suggested that the previous observations might be partially accounted for by this effect. However, the results following addition of fragmentin suggested a more interesting explanation.

Jones *et al.* (1990) demonstrated that target cells have the ability to repair damage induced by limited exposure to perforin. The effect of ATA on the DNA fragmentation induced by fragmentin in the presence of sub-lytic doses of perforin was assessed (Figure 5.6).

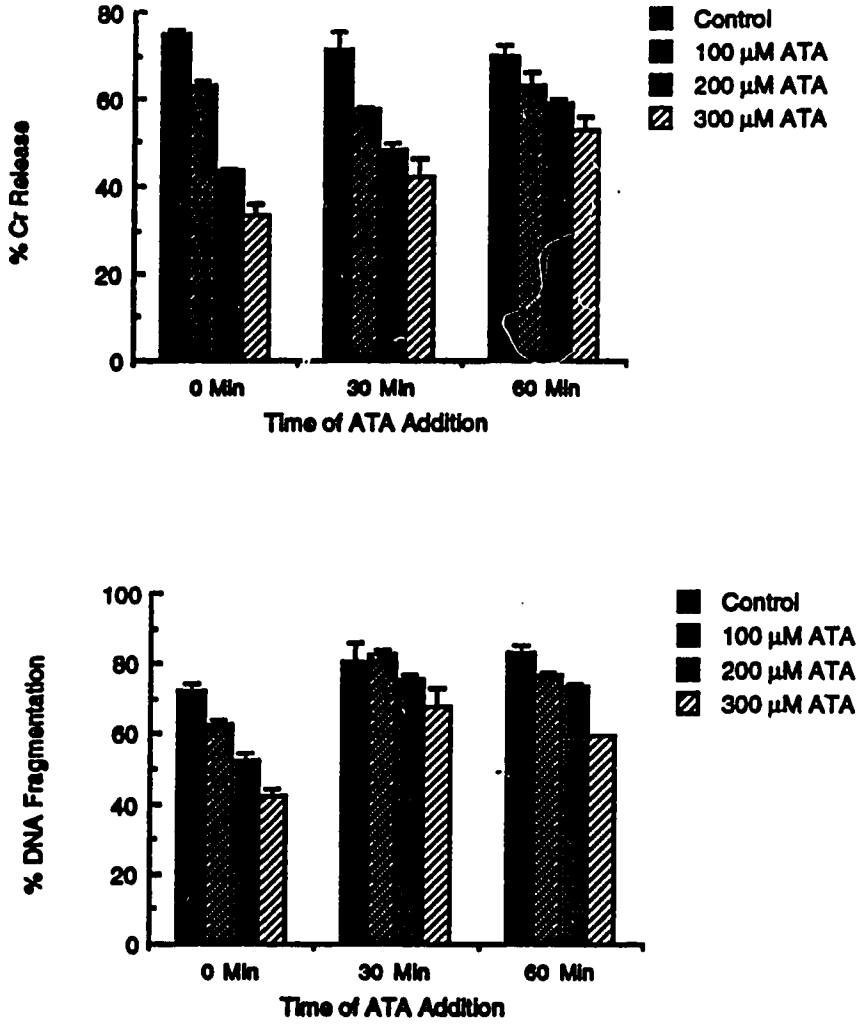


Figure 5.5: Addition of ATA following initiation of the lytic cycle inhibits lysis and fragmentation.

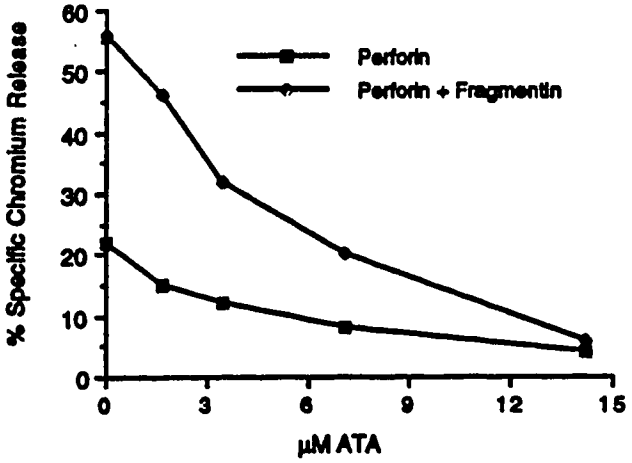
CTL, generated by incubation of mouse spleen cells with αCD3 mAb for 2 days, were incubated with appropriately labelled P815 targets at a 10:1 E:T ratio for 0, 30 or 60 minutes. At the various times, the indicated concentrations of ATA were added and incubations were continued. Fragmentation was assessed following a total incubation of 2 hours; cytotoxicity after 4 hours. Results are the mean ± SD of duplicate determinations.

Table 5.3: ATA inhibits the activity of purified perforin and fragmentin.

	Percent Specific Release	0 μM ATA	3 μM ATA	10 μM ATA
Perforin	^{51}Cr	58.0	17.9	0
	$^{125}IUdR$	0	4.0	4.0
Perforin + Fragmentin	^{51}Cr	72.8	38.5	0
	$^{125}IUdR$	59.2	37.8	23.7

YAC-1 target cells, double labelled with ^{51}Cr and $^{125}IUdR$, were incubated for four hours in the presence of perforin (0.8 μg/well) or perforin plus fragmentin (0.1 μg/well). Supernatants were collected for determination of release by gamma counting. Results are expressed as percent specific release calculated on a DMSO (-ATA) background.

A.



B.

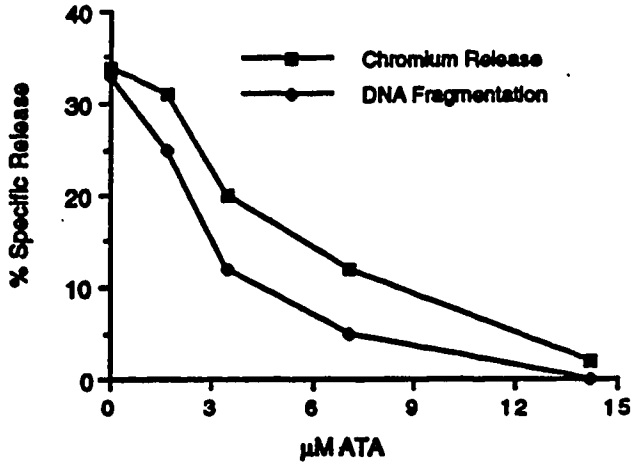


Figure 5.6: Fragmentin induces DNA fragmentation and chromium release. YAC-1 target cells, double-labelled with ^{51}Cr and $^{125}IUdR$, were preincubated with perforin (15 ng/ml) for 5 minutes; ATA and/or fragmentin (650 ng/ml) was then added and incubations were continued for a total of 2 hours. Results are expressed as percent specific release calculated on a DMSO (-ATA) background (Figure 5.6A). Figure 5.6B shows the specific release induced by fragmentin alone (i.e., (perforin + fragmentin) - perforin).

Figure 5.6a shows the specific chromium release induced by low levels of perforin alone or in combination with fragmentin. Even at the lowest concentration of perforin which permitted determination of the fragmentin effect, some chromium release was observed. However, addition of fragmentin resulted in a synergistic increase in target cell lysis. Again, this increase corresponded to the induction of DNA fragmentation by fragmentin. The relationship between the two events is most easily observed in Figure 5.6b. The level of DNA fragmentation directly correlated with the amount of chromium release induced by fragmentin alone (i.e., (perforin + fragmentin) - perforin). Furthermore, inhibition of both parameters by ATA occurred in a parallel manner suggesting that DNA fragmentation within the target cell directly leads to an appreciable level of cell death.

ATA specifically inhibits a nuclear endonuclease

In CTL-mediated lysis the endonuclease responsible for DNA fragmentation has not been localized to either the effector or the target, although the results of Ucker *et al.* (1992) suggest that it resides within the target cell. In a series of chromium release assays using either CTL or P815 targets pretreated with ATA there was no effect on lysis (Figure 5.7). Equally important was the observation that preincubation of either effector or target for four hours, the length of a standard chromium release assay, had no effect on viability as assessed microscopically by dye exclusion. Thus, in order to block killing ATA must be present during the assay. This result is consistent with a model of CTL-mediated lysis that involves the activation, by CTL, of an endonuclease within the target cell.

The ability of ATA to function as an endonuclease inhibitor in the isolation of nucleic acids (Hallick *et al.*, 1977) suggested that ATA was directly inhibiting the putative endonuclease responsible for DNA fragmentation. To test this possibility my collaborators in the laboratory of Doug Green (La Jolla, California) prepared nuclei from A1.1 cells, the T-cell hybridoma shown to undergo activation-induced apoptosis (Shi *et al.*, 1990). DNA fragmentation in these isolated

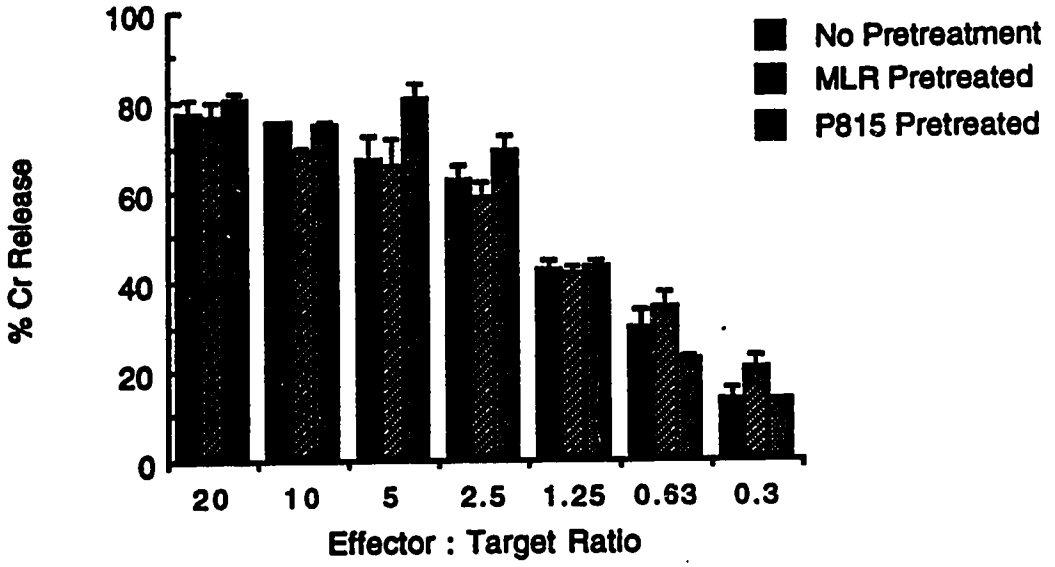


Figure 5.7: Pretreatment of effectors or targets with ATA has no effect on lysis.

MLR effectors or P815 targets were pretreated with 200 μM ATA for three hours at 37°C. Cells were washed twice with 50 ml RHFM and cytotoxicity assays were performed as described in Chapter 2. Results are the mean $\pm$ SD of triplicate determinations.

nuclei, induced by exposure to Ca^{++} and Mg^{++}, was effectively reduced by ATA (Figure 5.8), suggesting inhibition of a nuclear endonuclease responsible for DNA fragmentation.

Inhibition of chromium release, DNA fragmentation, and physiological cell death following programming for lysis.

Attempts to clarify the significance of apoptosis in CTL-mediated lysis have always resulted in confusion due to the high effector to target ratios used during *in vitro* killing assays. Although early results demonstrated the necessity for DNA fragmentation in target cell death mediated by isolated granular proteins, the relationship of these findings to *in vivo* cytolysis remained to be determined.

Two concerns arose from the studies utilizing purified granular proteins. The first concern was that those experiments were conducted using different target cells. It has been demonstrated that the role of DNA fragmentation in target cell lysis is dependent in part upon the nature of the target (Howell and Martz, 1987; Sellins and Cohen, 1991). The second concern was related to the methodology utilized. Quantitation of "total" fragmentation as done with the purified proteins, does not permit differentiation between release due to osmotic rupture (which would release all nonspecifically associated ^{125}IUdR) and release of fragmented DNA maintained within an intact cell.

To clarify these results, and to determine the effect of ATA in a physiologically relevant cell-cell system, the programming for lysis assay characterized in Chapter 4 was utilized. This experimental design resembles more closely the *in vivo* situation where contact between CTL and target is limited by either cell number (i.e., less than a 0.1:1 ratio of effectors to targets; Martz and Howell, 1989) or physical space.

Once the targets were programmed for lysis, they could be separated from the adherent killers and their subsequent fate followed. Results of one experiment are shown in Tables 5.4 and 5.5. Two modes of

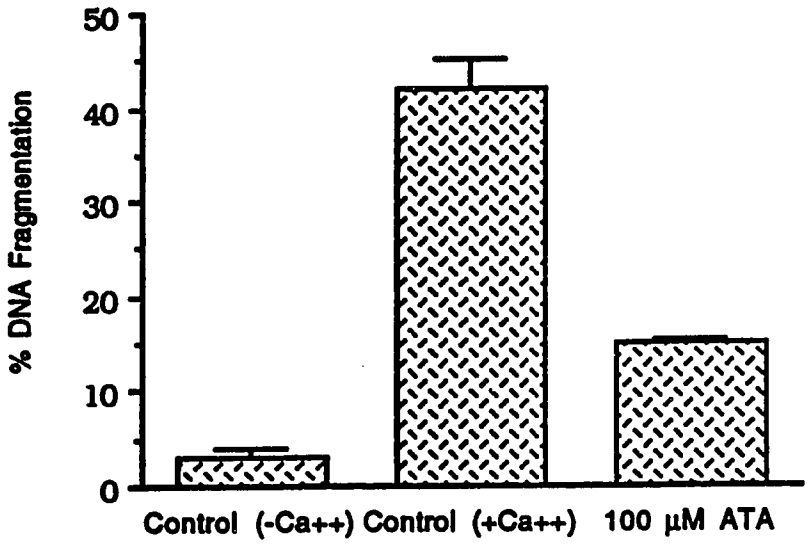


Figure 5.8: ATA inhibits DNA fragmentation in isolated nuclei.
Nuclei were isolated from the T cell hybridoma A1.1. Background levels of DNA fragmentation were measured in the presence of EDTA. DNA fragmentation was induced by the addition of Ca^{++} and Mg^{++}. Addition of 100 µM ATA significantly decreased DNA fragmentation.

Table 5.4: ATA inhibits apoptotic target cell death after programming for lysis.

Conjugation Time	Assay Conditions	Percent Cr Release	Percent Cytoplasmic DNA Fragmentation	^{3}H-Thymidine Incorporation
15 minutes	1.5 hrs - ATA	4.4 ± 0.1	20.3 ± 3.0	8660 ± 782
	1.5 hrs + ATA	3.3 ± 0.9	10.7 ± 0.1	13485 ± 129
	3.0 hrs - ATA	8.6 ± 1.8	14.1 ± 0.1	9863 ± 315
	3.0 hrs + ATA	7.1 ± 0.2	3.6 ± 0.3	13032 ± 870

Conjugation between CTL 21.9(IA) effectors and P815 targets was allowed to proceed for 15 minutes at an effector to target ratio of 10:1. Targets were then transferred to wells containing RHFM ± ATA and incubations were continued for varying lengths of time (Assay conditions). Chromium release, DNA fragmentation, and ^{3}H-thymidine incorporation were determined as outlined in Chapter 2. ^{51}Cr release and ^{3}H-thymidine incorporation results are the means (± SD) of triplicate determinations and DNA fragmentation is the average of duplicate determinations.

Table 5.5: The effect of ATA on osmotic target cell death after programming for lysis has occurred.

Conjugation Time	Assay Conditions	Percent Cr Release	Percent Cytoplasmic DNA Fragmentation	^{3}H-Thymidine Incorporation
30 minutes	1.5 hrs - ATA	36.6 ± 3.2	47.4 ± 1.6	5634 ± 43
	1.5 hrs + ATA	25.0 ± 1.7	25.2 ± 3.8	7423 ± 345
	3.0 hrs - ATA	44.1 ± 2.0	26.9 ± 0.1	6490 ± 509
	3.0 hrs + ATA	29.9 ± 3.9	3.4 ± 0.6	7566 ± 238

Conjugation between CTL 21.9(IA) effectors and P815 targets was allowed to proceed for 30 minutes at an effector to target ratio of 10:1. Targets were then transferred to wells containing RHFM ± ATA and incubations were continued for varying lengths of time (Assay conditions). Chromium release, DNA fragmentation, and ^{3}H-thymidine incorporation were determined as outlined in Chapter 2. ^{51}Cr release and ^{3}H-thymidine incorporation results are the means (± SD) of triplicate determinations and DNA fragmentation is the average of duplicate determinations.

target cell death could be distinguished depending upon the duration of contact which was permitted between the effector and the target. With a short conjugation time of 15 minutes (Table 5.4), perforin-mediated osmotic lysis was minimized as demonstrated by the low levels of chromium release observed at both the early and later assay periods. In contrast, there was a significant level of DNA fragmentation induced in the targets.

This DNA fragmentation corresponds to the percentage of total counts present within the cytoplasmic fraction of the labelled target. Lysis of the target releases these fragments into the supernatant with a resulting decrease in the percentage of fragmented DNA detected (cytoplasmic fragmentation). In order to avoid confusing DNA release due to lysis with fragmentation occurring within intact cells, only the cytoplasmic fraction was considered. ATA effectively inhibited the CTL-induced DNA degradation. More importantly, this inhibition resulted in a significant increase in the viability of target cells 24 hours later as measured by ^{3}H-thymidine incorporation.

In contrast, exposure for 30 minutes resulted in a significant level of perforin-mediated osmotic lysis, even at the earliest assay times (Table 5.5). As observed with the purified proteins, ATA partially inhibited this lysis. Again, there was a considerable amount of DNA fragmentation which was significantly inhibited by ATA. However, the ability of ATA to rescue target cells from osmotic lysis was dramatically reduced compared to that following fragmentation-induced lysis (compare cell viability in Tables 5.4 and 5.5). Therefore, in a cellular system which distinguishes between osmotic lysis and apoptosis, ATA prevents the physiological cell death of the programmed targets.

DISCUSSION

One of the hallmarks of apoptosis is fragmentation of the cellular DNA into a nucleosomal ladder (Wyllie *et al.*, 1980). In a similar manner, CTL-mediated cytolysis is accompanied by DNA degradation. In both cases, this type of internal disintegration precedes rupture of the cellular membrane. However, in neither instance has it been determined

whether DNA fragmentation directly leads to, or is simply a consequence of, lysis. This issue was examined using the nuclease inhibitor ATA.

ATA has been shown to be an effective inhibitor of apoptosis and the accompanying DNA fragmentation in thymocytes and in a T cell hybridoma (McConkey *et al.*,1989; Shi *et al.*, 1990). The results presented in this chapter demonstrate that CTL-mediated lysis can also be blocked by ATA in a dose-dependent manner (Figures 5.1 - 5.3). This effect was coupled with an inhibition of the DNA fragmentation which precedes lysis (Figure 5.4; Table 5.1). A similar observation was made by McConkey *et al.* (1990) using high levels of ATA to inhibit NK-induced target cell lysis. These authors determined that ATA did not interfere with the cytosolic calcium increase necessary for activation of the endonuclease responsible for DNA fragmentation, but they made no attempt to dissociate the two forms of target cell death (osmotic versus apoptotic) to determine the specificity of the ATA effect. Indeed, interpretation of many of the previous inhibitor studies is difficult due to a similar oversight. This point will be elaborated upon later.

One argument against apoptosis as a mode of target cell death is that apoptosis requires synthesis of DNA, RNA, and protein, while previous observations suggest that CTL-mediated lysis does not (Golstein *et al.*, 1991). One possible explanation for this apparent discrepancy between "programmed" and CTL-mediated apoptotic death is that the CTL directly introduces the molecules required for induction of the suicide pathway into the target cell. This would circumvent the need for transcription and translation in dying targets. Furthermore, Zychlinsky *et al.* (1991) demonstrated that pretreatment of target cells with inhibitors of macromolecular synthesis prior to exposure to CTL inhibits both cytolysis and DNA fragmentation. Perforin-mediated necrotic death was unaffected by these inhibitors. These observations strengthen the correlation between apoptosis and CTL-mediated lysis. Pretreatment of either effectors or targets with ATA prior to assay had no effect on cell lysis (Figure 5.7), suggesting that it does not interfere with the metabolic processes required for apoptosis. More importantly, this observation indicates that the endonuclease involved in target cell DNA

fragmentation is not activated and/or accessible until contact has been made with the CTL.

Cytolytic granules contain all the components required to achieve lysis of a target cell. Purification of the granule components necessary for lysis and DNA fragmentation in the target (Shi *et al.*, 1992b) enabled a closer examination of the ATA effect. The reduction in perforin-mediated lysis by ATA (Table 5.3) suggested that the CTL data could be interpreted in part on the basis of this inhibition. However, such a simple explanation was ruled out when fragmentin was included in the killing assay. Two significant points emerged from these experiments. Firstly, the inclusion of fragmentin significantly increased lysis as measured by the chromium release assay and secondly, ATA inhibited both the fragmentation-induced lysis as well as the DNA fragmentation itself.

Jones *et al.* (1990) demonstrated that cells have the ability to repair damage resulting from limited exposure to perforin alone. Attempts were made to differentiate osmotic lysis from apoptosis of the target cell using sub-lethal doses of perforin (Figure 5.6). Low levels of osmotic lysis were observed with limiting quantities of perforin, but addition of fragmentin induced high levels of DNA fragmentation which significantly increased cell lysis. Addition of ATA to the assay completely inhibited the DNA fragmentation as well as the corresponding chromium release. The level of ATA required to attain this level of inhibition with the purified proteins was significantly lower than when CTL were used as effectors. Although the site of action is the target cell, both the CTL and the target can take up ATA. Therefore, in the presence of a tenfold excess of effector cells, higher concentrations of ATA are necessary to achieve the same concentration within the target cell. In this artificial environment osmotic and apoptotic lysis were separated and ATA was shown to be an effective inhibitor of both.

As mentioned previously, the role of apoptosis in target cell lysis has been questioned due to an inability to separate the two modes of cell death. Purification of perforin and the observation that it is capable of inducing significant target cell osmolysis led to the

suggestion that this is the primary mechanism of CTL-induced death (Tschopp and Nabholz, 1990). However, evidence suggests that the *in vivo* situation is more complicated. Berke and Rosen (1987) demonstrated significant cytolysis by *in vivo* primed peritoneal exudate lymphocytes in the absence of hemolytic (i.e., perforin) activity. Furthermore, perforin alone is not capable of inducing the controlled, pre-lytic DNA degradation observed in target cells following CTL attack (Duke *et al.*, 1983; Russell, 1983). Any study of the role of DNA fragmentation in target cell lysis must therefore separate osmotic (perforin-mediated) rupture from other modes of death. This type of separation was accomplished using the purified proteins.

A similar separation was observed using a physiologically relevant experimental model i.e., live cells. The lytic event can be divided into a number of discrete stages (Martz, 1975) including conjugate formation, delivery of the lethal hit ("programming"), and target cell death. Only the first two stages require the participation of the CTL. The use of an adherent CTL line, as described in Chapter 4, made it possible to carefully control the first two steps and to subsequently monitor the fate of the target cell.

Target cells were programmed for lysis in the absence of ATA and then removed from the adherent effectors. Their fate was examined, in the presence or absence of ATA (Tables 5.4 and 5.5), with regard to lysis (^{51}Cr release), DNA fragmentation ($^{125}IUdR$ release), and cell viability (^{3}H-thymidine incorporation). When conjugate formation times were kept short (i.e., 15 minutes) to minimize perforin-mediated osmotic lysis, 200 μM ATA resulted in significant inhibition of DNA fragmentation and chromium release. Most importantly, this was reflected in an increase in target cell viability. Thus, T-cell mediated death comprises two components, osmotic lysis (necrosis) mediated by perforin and functional cell death resulting from fragmentation of DNA (apoptosis). Both processes are inhibited by ATA.

CTL-mediated lysis and activation-induced T cell death are both preceded by DNA fragmentation, suggesting a common point in both pathways leading to lysis. The results presented in this chapter

suggest a mechanism for cell death in both systems that intersects at a common ATA-sensitive step. Furthermore, the activity of ATA as an inhibitor of endonucleases (Hallick *et al.*, 1977) suggests that these pathways converge at this point. It is tantalizing to speculate that fragmentation actually leads to cell death and that ATA mediates its effect by inhibition of a nuclease common to both pathways. Although the endonuclease has not been identified, ATA was shown to inhibit its Ca^{++}-Mg^{++} dependent activity in isolated nuclei (Figure 5.8).

The argument often used against this model is that CTL can lyse anucleate red blood cells and therefore no DNA fragmentation is required. However, the lysis of red blood cells can be readily achieved by perforin alone, while this important component of the lytic machinery does not induce fragmentation in nucleated cells. Clearly, another component of the lytic granules is required to achieve physiologically relevant lysis and the hemolytic activity is only indicative of the presence of perforin.

The same argument applies to the situation described by Duke and colleagues (1989), in which cytotoxic granules caused lysis but not DNA fragmentation in target cells, again suggesting that the former does not depend on the latter. However, the quantity of granules required to produce significant lysis without DNA fragmentation corresponded to approximately 100-500 times the number of CTL required to produce the same amount of lysis with fragmentation. This point is especially important considering that effector to target ratios *in vivo* are probably less than 1:1. It is therefore possible that DNA fragmentation is a major contributor to cell death, but that at a high enough concentration, other mediators (such as perforin) can kill cells without inducing apoptosis. In support of this suggestion, Nishioka and Welsh (1992) demonstrated that at high effector to target ratios it was possible to inhibit DNA fragmentation without altering target cell lysis.

The results presented in this chapter suggest that CTL possess the capability to lyse their targets by more than one mechanism. At the higher effector to target ratios commonly used *in vitro*, the effect of

DNA fragmentation on target cell viability may be masked by the perforin-mediated osmotic lysis. *In vivo*, where high effector to target ratios are not possible, induction of apoptosis in the target cell may be critical for its destruction. The programming for lysis assay (Tables 5.4 and 5.5) argues in favour of CTL possessing at least two functional methods for target cell destruction.

Further support for the existence of two discrete mechanisms by which CTL can induce target cell death is provided by Cosgrove *et al.* (1991). These authors demonstrated that although the majority of CTL clones produced both membrane and DNA damage in their target cells, certain clones had the ability to induce one without the other. In addition, the contribution of each of the two modes of target cell damage varied depending upon the effector to target ratio used.

These results, taken together with those of McConkey and colleagues (1989; 1990) suggest that apoptosis induced in T cells by glucocorticoids, A23187, or activation through the T cell receptor, or in target cells by CTL and NK share a common ATA-inhibitable mechanism. Based on the observations with isolated nuclei, the prime candidate for this common mechanism is the Ca^{++}-Mg^{++}-dependent endonuclease associated with DNA fragmentation. The results presented in this chapter support the idea that DNA fragmentation is a necessary step in both apoptosis and CTL-mediated killing.

In summary, CTL-mediated target cell lysis can be achieved by a combination of at least two pathways, osmotic lysis and DNA fragmentation. The relative contributions of the two are controlled by the sensitivity of the targets to each lytic mechanism and by the concentrations of the relevant effector molecules. These, in turn, are influenced by the contents of the CTL granules and the effector to target ratios used. Thus, in a situation where there is a high concentration of perforin, osmotic lysis is the overwhelming form of death, and no amount of inhibition of DNA fragmentation is effective. The data presented in this chapter clearly indicates that DNA fragmentation can lead to cell death when the perforin effect is less pronounced. Although the relative contributions of these two pathways

in vivo is unclear, in many instances target cells will certainly outnumber the effectors. It is therefore suggested that the apoptotic pathway is of great importance in such circumstances. This would certainly explain the large number of anecdotal reports of *in vivo* cell-mediated immune responses which occur in the absence of morphological evidence for osmotic lysis.

CHAPTER 6: INVESTIGATION OF THE ROLE OF CYTOTOXIC CELL PROTEINASES IN CTL-MEDIATED LYSIS AND DNA FRAGMENTATION

INTRODUCTION

Target cell lysis mediated by either cytotoxic T lymphocytes (CTL) or natural killer (NK) cells is a complicated process. Cell death is usually, but not always, preceded by degradation of the chromatin into a nucleosomal ladder (Duke *et al.*, 1983; Russell, 1983). Perforin alone induces osmotic lysis, but other molecules must be involved in initiating the DNA damage. CTL-specific serine proteinases, designated cytotoxic cell proteinases (CCPs; Bleackley *et al.*, 1988b) or granzymes (Masson *et al.*, 1986a), are potential candidates for this role.

Early studies with various serine proteinase inhibitors suggested the involvement of CCPs in the lytic mechanism prior to their identification (Chang and Eisen, 1980; Redelman and Hudig, 1980; Hudig *et al.*, 1981). Initially they were thought to be membrane-bound proteins involved in the early stages of transmembrane signalling (Pasternack *et al.*, 1983; Lavie *et al.*, 1985; Utsunomiya and Nakanishi, 1986). Further investigation revealed that these proteinases were located within the perforin-containing cytolytic granules (Redmond *et al.*, 1987; Ojcius *et al.*, 1991).

The importance of the early inhibitor studies became apparent when a family of CTL-specific serine proteinases was isolated from cytolytic granules. Pasternack and Eisen (1985) identified a protein in CTL with high levels of activity against the trypsin substrate BLT. This protein was identified as granzyme A or Hanukah factor (HF) (Masson *et al.*, 1986a; 1986b; Young *et al.*, 1986). The remainder of the granzyme family was isolated by Masson and Tschopp in 1987. Meanwhile, differential and subtractive hybridization techniques were employed to identify the corresponding cDNA clones. Gershenfeld and Weissman (1986) isolated the cDNA corresponding to HF. Lobe *et al.* (1986a; 1986b) found the first sequences corresponding to members of the CCP family: CCP1 and CCP2. The remainder of the cDNAs were subsequently isolated (Bleackley *et al.*, 1988a; 1988b; Jenne *et al.*, 1988a, 1988b; Kwon *et al.*, 1988).

Seven granzymes have now been identified in the mouse and four in humans.

The CCPs are preferentially expressed by $CD8^+$ CTL, although mRNA has also been detected in $CD4^+$ killer cells (Kramer *et al.*, 1986; Garcia-Sanz *et al.*, 1990; Lancki *et al.*, 1991). One requirement for a protein involved in the lytic process is that its expression should correlate with cytolytic activity. CCP1 and CCP2 mRNA levels increase prior to maximal cytotoxicity in mitogen (Concanavalin A) stimulated mouse splenocytes (Bleackley *et al.*, 1988a). Similarly, CCP1 expression precedes cytolytic activity following activation with either antigen or an αCD3 mAb (Prendergast *et al.*, 1992b). *In vivo*, expression of both HF and CCP1 are observed in situations, such as allograft rejection, where CTL are involved (Mueller *et al.*, 1988a; 1988b).

Although the CCPs have been extensively characterized, their role in CTL-mediated lysis has remained elusive. It is possible that the proteinases may be involved in mediating both membrane damage and DNA fragmentation. The observation that perforin induces lysis in the absence of any other mediators suggested that the proteinases play a secondary role in the lytic pathway (Tschopp and Nabholz, 1990; Podack *et al.*, 1991). However, several proteolytic activities co-purify with perforin, suggesting that the proteinases may be involved in its activation (Hudig *et al.*, 1992). Others have postulated that the proteinase family functions as a proteolytic cascade (Gershenfeld *et al.*, 1988) similar to complement.

One major problem in defining the role of the proteinases has been the inability to identify their natural substrates. Progress has recently been made in this regard. Granzyme A cleaves nucleolin (Pasternack *et al.*, 1991), as well as fibrin, casein, and extracellular matrix proteins (Simon *et al.*, 1987). Synthetic substrates have been utilized to characterize the specificities of the other family members (Odake *et al.*, 1991). The enzymatic activities present in cytolytic granules can be categorized as follows: 2 tryptases (granzyme A and granzyme D), 2 chymases, 1 aspase corresponding to CCP1 (granzyme B) as predicted by molecular modelling (Murphy *et al.*, 1988), 1 metase, and 1

serase (Hudig *et al.*, 1991; Odake *et al.*, 1991). No substrate has been identified for CCP2 (granzyme C).

Evidence has accumulated for the involvement of serine proteinases in target cell DNA fragmentation (Hayes *et al.*, 1989; Shi *et al.*, 1992a, 1992b). Purified proteinases alone are incapable of inducing this damage. However, prior permeabilization of cells with Triton X-100 or sublytic doses of perforin results in significant levels of proteinase-mediated DNA fragmentation. Further evidence for this suggestion is provided by Shiver *et al.* (1992) who transfected a rat basophilic leukemia cell line with perforin and/or granzyme A constructs. Expression of perforin alone resulted in lysis of targets cells, at low efficiency, with no DNA fragmentation. Co-expression of both proteins resulted in increased target cell lysis accompanied by DNA fragmentation. The mechanism by which the proteinases induce this damage has not been elucidated. There may be a direct effect on the nuclease involved or the CCPs may modulate the activity of other proteins involved in initiating DNA degradation.

For example, TIA-1 is a CTL granule protein indirectly involved in DNA fragmentation. Sequence analysis reveals a potential serine proteinase cleavage site (Tian *et al.*, 1991). CTL-specific proteinases may gain access to the target cell via perforin channels. Cleavage of TIA-1 might activate it to induce DNA degradation. Further characterization of this protein is required to determine the validity of this proposal.

The experiments outlined in this chapter were designed to investigate the role of the CCPs in both CTL-mediated lysis and DNA fragmentation. Experiments with three serine proteinase inhibitors, N-acetyl-L-tyrosine ethyl ester (ATEE), N-benzoyl-L-arginine ethyl ester (BAEE), and 3,4-dichloroisocoumarin (DCI) are presented. DCI was added in combination with the endonuclease inhibitor aurintricarboxylic acid (ATA) to evaluate the relationship between proteinase activity and the endonuclease activity involved in target cell DNA fragmentation. The role of the CCPs during the KCIL stage was investigated using the programming for lysis assay (described in Chapter 4).

Although results from a number of laboratories suggest that these inhibitors block the activities of purified proteinases, as well as cytolytic granules, no evidence has been presented for their effects on live cells. The results presented in this chapter suggest a dual role for the proteinases in the lytic mechanism.

RESULTS

Proteinase inhibitors modulate cytolytic activity to varying degrees

Three inhibitors were chosen to examine the role of the proteinases in the lytic mechanism. N-acetyl-L-tyrosine ethyl ester (ATEE) and N-benzoyl-L-arginine ethyl ester (BAEE) are competitive substrate inhibitors of chymotrypsin- and trypsin-like enzymes respectively (Kaiser and Hoskin, 1992). Both are reversible inhibitors which compete with the natural substrate for binding in the active site. The third compound, 3,4-dichloroisocoumarin (DCI), is a mechanism-based, general serine proteinase inhibitor (Harper *et al.*, 1985) described as irreversible, specific, and rapidly reactive. It remains inactive until the isocoumarin ring is enzymatically opened resulting in acylation of the active site serine residue of the proteinase. Subsequent alkylation of the histidine residue may follow. Acylation can be reversed over a period of several hours or days; alkylation is irreversible (Hudig and Powers, 1992).

Various concentrations of these inhibitors were used to modulate the cytolytic activity of αCD3 mAb-activated CTL. The trypsin substrate BAEE (Figure 6.1) was ineffective at preventing lysis, although 10 mM BAEE did cause low levels of inhibition. In contrast, all three concentrations of the chymotrypsin substrate ATEE significantly blocked chromium release (Figure 6.2). DCI was considerably more effective than either of the other two inhibitors (Figure 6.3). For example, 40 μM DCI completely inhibited lysis at a 10:1 E:T ratio, while the highest concentration of ATEE decreased lysis by approximately 70% and BAEE by only 25%. The significance of these observations will be discussed later.

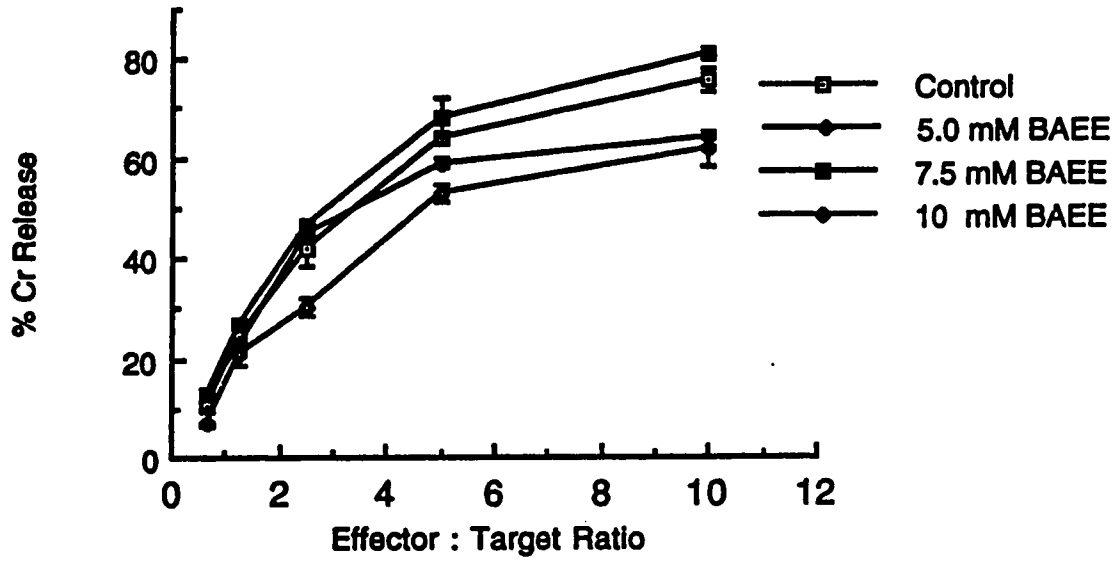


Figure 6.1: BAEE does not prevent chromium release.

Various concentrations of BAEE were added to αCD3 mAb-stimulated spleen cells in a 96-well V-bottom plate immediately prior to addition of targets. Chromium release assays involved 4 hour incubations. Cytolytic activity was then determined as described in Chapter 2. Results for BAEE are expressed on an ethanol background where % specific release is determined by the formula: (% release + BAEE) x (% control release - drug) divided by (% release in the presence of an equivalent amount of solvent for the drug). All points are the mean ± SD of triplicate determinations.

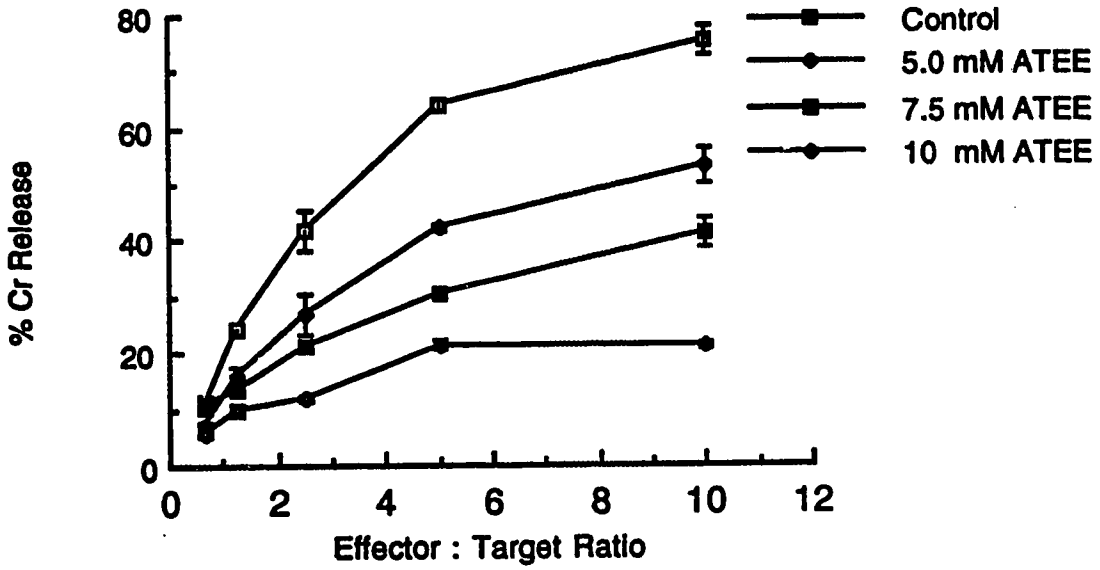


Figure 6.2: ATEE inhibits chromium release in a dose-dependent manner. Experimental conditions are similar to those described in the legend to Figure 6.1.

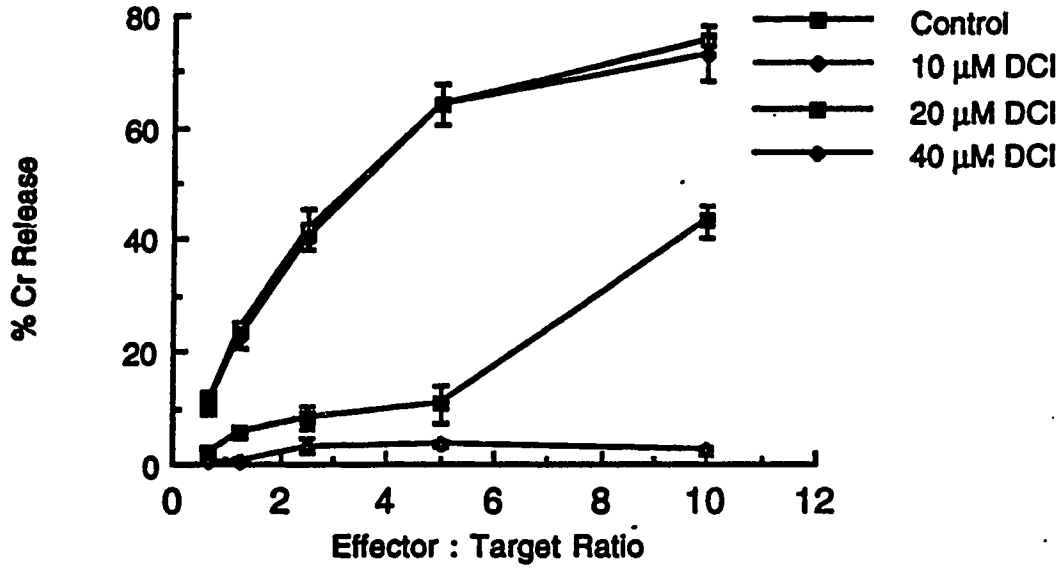


Figure 6.3: DCI completely blocks CTL-mediated lysis.
Experimental conditions are outlined in the legend to Figure 6.1. Results for DCI are expressed on a DMSO background.

Proteinase inhibitors block DNA fragmentation

CTL-induced DNA fragmentation was also examined in the presence of these inhibitors. A summary of the results is presented in Table 6.1. BAEE, as seen with chromium release, did not significantly alter the extent of DNA degradation. ATEE exhibited a dose-dependent inhibition of fragmentation which correlated with its effects on chromium release. The most pronounced inhibition was seen with DCI. In addition, the pattern of inhibition differed from that of the other two. For example, a significant reduction in chromium release (i.e., 43%) was observed with 20 μM DCI, but DNA fragmentation was inhibited by only 13%. This observation suggested that different proteinases, with varying sensitivity to DCI, were involved in the two processes.

DCI plus ATA produces greater inhibition

The role of the CCPs in DNA fragmentation was further investigated using DCI in combination with the endonuclease inhibitor ATA (described in detail in Chapter 5). Both agents alone significantly reduced lysis at all E:T ratios (Figure 6.4). When used together, the inhibitory effect was additive. Inhibition of DNA fragmentation was also additive (Table 6.2).

Several conclusions arise from these observations. The first is that the CCPs are involved in both DNA fragmentation and membrane damage. Secondly, the results suggest that DNA damage must be controlled at two levels. The first level is blocked by addition of the proteinase inhibitor, while the second is blocked by ATA. If both proteinase and endonuclease were involved in the same pathway, inhibition would be less than additive.

DCI blocks lysis, but not fragmentation, when added after programming for lysis

The programming for lysis assay described in Chapter 4 was used to examine the effect of DCI, in the presence or absence of ATA, on the KCIL stage of the lytic cycle. The results of a representative experiment following both 15 and 30 minutes of programming were similar

Table 6.1: Inhibition of DNA fragmentation by the serine proteinase inhibitors.

Treatment	% DNA Fragmentation	% Chromium Release
Control	41.4 ± 0.8	75.4 ± 2.4
5 mM BAEE	38.3 ± 5.1	63.8 ± 1.6
7.5 mM BAEE	36.0 ± 2.4	80.3 ± 1.1
10 mM BAEE	31.6 ± 3.7	61.2 ± 4.4
Control	41.4 ± 0.8	75.4 ± 2.4
5 mM ATEE	23.8 ± 2.8	53.0 ± 1.6
7.5 mM ATEE	18.1 ± 2.0	41.2 ± 3.8
10 mM ATEE	9.7 ± 4.9	21.3 ± 0.8
Control	41.4 ± 0.8	75.4 ± 2.4
10 μM DCI	35.5 ± 1.1	72.9 ± 4.6
20 μM DCI	35.8 ± 1.6	43.3 ± 2.7
40 μM DCI	5.6 ± 0.6	2.5 ± 0.8

«CD3 mAb-stimulated spleen cells were incubated with various concentrations of the inhibitors for 2.5 hours at an E:T of 10:1. DNA fragmentation was determined as described in Chapter 2. Results for ATEE and BAEE are expressed on an ethanol background, DCI on a DMSO background, as described in the legend to Figure 6.1. Values are the mean ± SD of duplicate determinations. Chromium release results at a 10:1 E:T are presented for comparison.

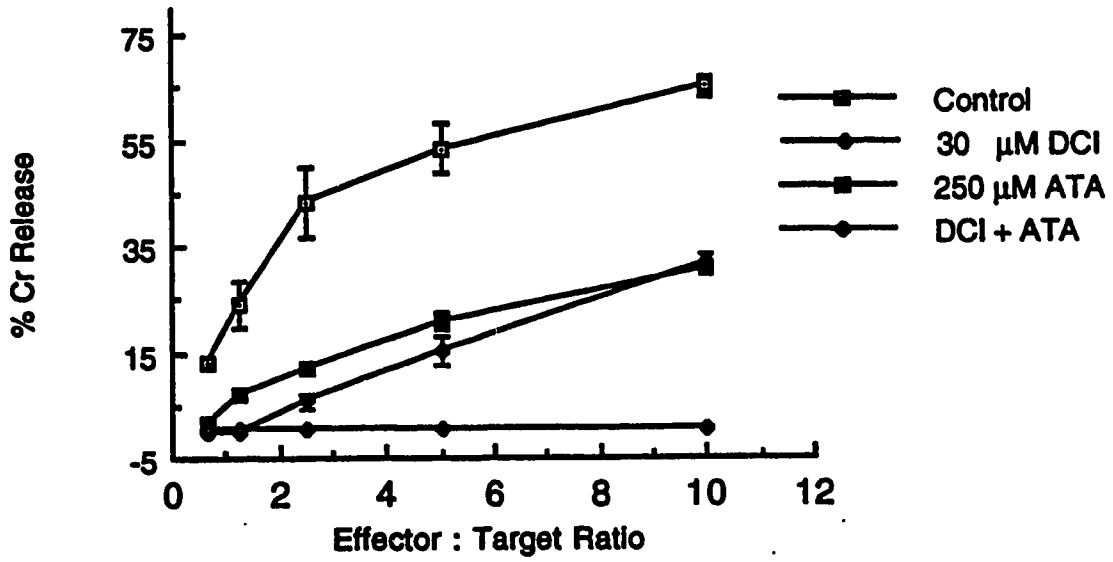


Figure 6.4: The combination of ATA and DCI prevents chromium release completely.

P815 targets were incubated with CTL 21.9(I) effectors for four hours in the presence or absence of the inhibitors. Chromium release was determined as described in Chapter 2. Results with DCI are presented on a DMSO background as explained in the legend to Figure 6.1. Each point on the curve represents the mean ± SD of triplicate determinations.

Table 6.2: Additive inhibition of DNA fragmentation by DCI and ATA.

Treatment	% DNA Fragmentation
Control	42.0 ± 3.6
30 μM DCI	16.4 ± 7.7
250 μM ATA	19.5 ± 3.1
DCI + ATA	5.4 ± 1.5

Results presented in this table are the mean ± SD of 2 separate experiments and are representative of 4 similar determinations. CTL 21.9(I) effectors were incubated with P815 targets at a 5:1 E:T for 1.5 hours and specific release was determined as described in Chapter 2. Results for DCI are presented on a DMSO background as outlined in the legend to Figure 6.1.

(Table 6.3). DCI induced little inhibition of chromium release. In contrast, ATA significantly prevented lysis when added following programming. Inhibition of lysis was additive when ATA and DCI were present from the start of the assay. Inclusion of the combination following programming resulted in a more pronounced reduction than when either was present alone.

DCI had no effect on DNA fragmentation, regardless of whether it was added following 15 or 30 minutes of programming. ATA effectively blocked DNA damage in both instances. Most importantly, the combination of ATA plus DCI was no more inhibitory than ATA alone. This further confirms that DCI has no effect on DNA fragmentation when added at a later time.

These results with the programming for lysis assay suggest that the proteinases are involved in two discrete steps of the lytic process. Inhibition of chromium release, but not DNA fragmentation, following programming indicates that the DCI-sensitive proteinase completes its role in DNA damage within the 15-30 minutes before DCI is added. The involvement of the other proteinase(s) in membrane damage continues during the KCIL phase.

DISCUSSION

BAEE did not prevent either CTL-induced chromium release or DNA fragmentation. ATEE and DCI effectively inhibited both. When the proteinase inhibitor DCI and the endonuclease inhibitor ATA were added together at the beginning of the assay they completely blocked both lysis and DNA damage. In contrast, using the programming for lysis assay, DCI alone inhibited cytotoxicity to a limited extent with no effect on DNA fragmentation. ATA, as demonstrated previously, reduced both parameters. The combination of ATA plus DCI blocked lysis to a greater extent than either alone. Fragmentation, on the other hand, was inhibited to the level observed when only ATA was added.

Both ATEE and BAEE compete with the natural substrates for the proteinase binding sites. BAEE, preferentially recognized by enzymes with a trypsin-like specificity, did not prevent lysis or fragmentation

Table 6.3: DCI does not prevent DNA fragmentation following programming for lysis.

Treatment	15 Minutes		30 Minutes	
	^{51}Cr	$^{125}IUdR$	^{51}Cr	$^{125}IUdR$
Control	20.7 ± 4.3	12.3 ± 0.2	23.2 ± 1.6	25.7 ± 1.1
15 μM DCI	20.4 ± 2.2	12.5 ± 0.4	20.6 ± 0.5	24.5 ± 0.5
DCI + ATA	4.6 ± 3.0	10.3 ± 0.1	10.6 ± 1.4	9.6 ± 1.1
30 μM DCI	19.8 ± 2.6	11.6 ± 0.7	16.0 ± 1.5	23.5 ± 1.6
DCI + ATA	0.6 ± 1.4	8.9 ± 0.5	5.3 ± 0.8	8.2 ± 0.9
45 μM DCI	12.8 ± 2.2	10.8 ± 1.3	18.5 ± 1.4	24.5 ± 0.2
DCI + ATA	0.1 ± 0.1	10.5 ± 0.4	7.9 ± 1.6	7.2 ± 2.0
250 μM ATA	6.5 ± 1.7	8.5 ± 0.1	14.5 ± 2.7	9.4 ± 0.6

P815 targets were programmed for lysis by incubation with CTL 21.9(IA) for 15 or 30 minutes at a 10:1 E:T as described in Chapter 2. Results in this table are the mean ± SD of duplicate determinations of DNA fragmentation ($^{125}IUdR$) measured 2 hours following programming and six determinations of lysis (^{51}Cr) measured 5 hours following programming. These results are representative of 6 similar experiments.

at the concentrations utilized. Higher doses caused problems with both drug solubility and toxicity to the targets. The results with this inhibitor suggest that the trypsin-like proteinases, granzymes A and D (Odake *et al.*, 1991), are not involved in either the lysis or fragmentation measured in these assays.

This simple explanation seems unlikely for a number of reasons. Granzyme A is a tryptase which has been implicated in DNA fragmentation using permeabilized target cells (Hayes *et al.*, 1989) and cells subjected to sublethal doses of perforin (Shi *et al.*, 1992b). Similarly, transfection of rat basophilic leukemia cells with both perforin and granzyme A expression vectors enables them to lyse tumor target cells with DNA fragmentation (Shiver *et al.*, 1992). In addition, antisense expression vectors which inhibit protein expression result in a significant, though incomplete, reduction in the levels of both chromium release and DNA fragmentation (Talento *et al.*, 1992).

A more likely explanation for the absence of inhibition using BAEE is that the role of the tryptases is dependent upon the nature of both the CTL and the target cell. It has been shown that various target cells are differentially sensitive to the effects of individual proteinases (Shi *et al.*, 1992b). In addition, expression of the CCP family members is dependent upon the nature of the stimulus activating the CTL (Prendergast *et al.*, 1992b). Thus, the lack of affect in these experiments may reflect the involvement of other lytic mechanisms which are independent of tryptase activities.

ATEE, a substrate inhibitor for chymotrypsin-like proteinases, significantly reduced both chromium release and DNA fragmentation in a dose-dependent manner. Several previous results suggest that this is a specific effect. First, ATEE does not inhibit effector-target conjugate formation, even at the highest concentration used (Kaiser and Hoskin, 1992). Furthermore, at least one of the chymases is required for granule-induced chromium release (Hudig *et al.*, 1989). ATEE inhibited DNA fragmentation to a greater extent than chromium release at all concentrations tested, suggesting that the chymases are predominantly involved in this process. However, it has not been determined which of

the CCPs are responsible for the chymase activities (Hudig *et al.*, 1991).

ATEE, as well as BAEE, are reversible inhibitors. This makes interpretation of chromium release results somewhat difficult. It is possible that they slow the rate of lysis without actually preventing it (Hudig and Powers, 1992). High concentrations of these drugs may be necessary to inactivate a major portion of the proteinases involved at any one time.

An irreversible inhibitor was used to overcome the potential problems of reversibility and to further clarify the role of the serine proteinases in target cell death. DCI is a specific, mechanism-based inhibitor activated by interaction with the active site serine residue (Harper *et al.*, 1985). It inhibits the activities of all of the CCPs, albeit with differing efficiencies (Odake *et al.*, 1991). For example, it is the most potent inhibitor of the CCP1 aspase activity (Poe *et al.*, 1991), but has less affect on the activity of the tryptases. DCI also inhibits the lysis mediated by isolated cytolytic granules (Hudig *et al.*, 1991) and does not interfere with the activity of purified perforin (Shi *et al.*, 1992a).

DCI inhibited both chromium release and DNA fragmentation at considerably lower concentrations than ATEE. There are two possible reasons for this observation. First, it may be partially explained by the irreversible nature of DCI. In addition, DCI inhibits the activity of all the CCPs, not just the chymases (Hudig *et al.*, 1989).

Chromium release was inhibited to a greater extent at lower concentrations of DCI than was DNA fragmentation. Ultimately, however, both processes were virtually completely blocked. This was the first indication that the serine proteinases were involved in several aspects of CTL-mediated lysis. It appears that CCPs with a greater sensitivity to DCI induce the membrane damage measured by chromium release. Other proteinases, less sensitive to DCI, initiate DNA fragmentation. This suggests that each proteinase has a specific function in the lytic process.

This hypothesis is supported by observations of CCP expression in response to different stimuli. CCP1 is expressed prior to the peak of cytolytic activity in splenocytes activated with antigen, mitogen, or αCD3 mAb (Bleackley *et al.*, 1988a; Prendergast *et al.*, 1992b). CCP2 mRNA is also present before cytotoxicity following antigen or mitogen, but not αCD3, stimulation. CCP3, CCP4, and CCP5 are not detected at all following αCD3 activation. Thus, different proteinases are produced at various stages of CTL activation, suggesting varying roles. In addition, it has been observed that different target cells vary in their sensitivity to induction of DNA fragmentation by the rat CCP homologs, Fragmentins 1,2 and 3 (Shi *et al.*, 1992a). The expression of a variety of serine proteinases thus enables CTL effectors to destroy numerous targets.

Further evidence supporting a dual role for the proteinases in the lytic mechanism was obtained by using DCI in combination with ATA. ATA significantly reduced DNA fragmentation by inhibiting the responsible endonuclease. Even in the presence of ATA, DCI further prevented DNA damage. Presumably, one or more of the CCPs is involved in an early stage of the fragmentation process i.e., before the endonuclease. The inability of DCI alone to completely block fragmentation suggests that several pathways activate this enzyme.

Addition of the proteinase inhibitors during the KCIL stage partially prevents lysis (Brogan and Targan, 1986). This observation was re-assessed using the programming for lysis assay characterized in Chapter 4. Addition of DCI during KCIL inhibited lysis, both alone and in conjunction with ATA. In contrast, there was no affect on DNA fragmentation. The inherent variability of the chromium release measurements at early time points made it difficult to assess the magnitude of inhibition. However, in all experiments a similar effect on both chromium release and DNA fragmentation was observed. Some of the CCPs appear to be involved only in the induction of the fragmentation pathway. Once this pathway is initiated the proteinases continue to function in other aspects of target cell lysis. Perforin activity is not involved in the inhibition since the CTL are no longer

present during the incubation with DCI and perforin is quickly inactivated in the presence of calcium if it is not already inserted into the membrane. Furthermore, DCI does not interfere with the function of perforin (Shi *et al.*, 1992b).

The results presented in this chapter suggest a dual role for the CCPs in CTL-mediated target cell death. They are rapidly involved in the initiation of DNA fragmentation in, and have a continuing role in the actual lysis of, the target cell. It was not possible to determine if different proteinases were involved in each process or if all proteinases could participate in both. Specific inhibitors of each proteinase should make it possible to examine their individual roles in the lytic pathway.

In summary, tryptases do not play a significant role in the short-term DNA fragmentation or chromium release assays used in these experiments. ATEE results suggest a role for the chymases, activities not yet associated with a particular CCP, in both processes. DCI confirmed a role for the CCPs in the two mechanisms and extended this observation. The proteinases are involved only during the early stages of DNA fragmentation. In contrast, they have a continuing function in the membrane damage which results in chromium release. These observations are summarized in the model presented in Chapter 7.

CHAPTER 7: CONCLUDING REMARKS

SUMMARY

All the studies described in this thesis were directed toward the goal of developing a greater understanding of the mechanism(s) by which CTL destroy target cells. The general discussion which follows is designed to provide an overview connecting the experimental results.

The generally accepted model of target cell lysis involves a directed release of cytolytic granules from the CTL toward the bound target cell (Henkart, 1985; Young, 1989; Podack and Kupfer, 1991). The molecules contained within the granules, perforin and the CCPs, are involved in cell lysis. CTL can also efficiently lyse targets in the absence of degranulation (Ostergaard *et al.*, 1987; Trenn *et al.*, 1987). PEL cells, generated *in vivo*, do not appear to express either hemolytic (perforin) or BLT esterase (representative of granzyme A) activity (Berke *et al.*, 1987; Dennert *et al.*, 1987), yet they are potent cytolytic effectors. However, detection of perforin and CCP mRNA in these cells makes this result controversial (Brunet *et al.*, 1987; Nagler-Anderson *et al.*, 1989; Garcia-Sanz *et al.*, 1990; Ebnet *et al.*, 1991). These observations suggest the existence of alternate mechanisms of target cell lysis which are independent of cytolytic granules and their contents. These issues were all addressed in the experiments presented in this thesis.

One difficulty in assessing the significance of the reports regarding perforin and CCP expression in freshly isolated PEL is the likelihood of contamination by populations of cells such as NK which do, in fact, express these molecules. Studies utilizing cytolytic hybridomas to determine if lysis can be achieved in the absence of perforin and the CCPs provide several advantages. They are a pure population of cells which have been well-characterized and do not require IL2 for growth (Kaufmann *et al.*, 1981; Kaufmann and Berke, 1983). This last point is important as IL2 has been shown to induce expression of both perforin and the CCPs in primary PEL cells (Berke and Rosen, 1988).

PCR analysis with total RNA isolated from five cytolytic hybridomas revealed no significant expression of either perforin or the CCPs. In spite of this, some of the hybridomas were capable of effectively mediating antigen-specific lysis. Characterization of the lytic process revealed that a unique system was employed to induce target cell death. Specific receptor-mediated contact was required for lysis. In contrast to CTL-mediated death, DNA fragmentation induced by the hybridomas accompanied, but did not precede, lysis. Furthermore, protein synthesis was required during the lytic cycle induced by the hybridomas. Experiments with inhibitors of poly(ADP-ribosyl)ation implicated activation of poly(ADP-ribose)synthetase in the lytic process. Similar observations have been made in TNF-mediated cytolysis (Agrawal *et al.*, 1988; Vassali, 1992), but there was no evidence to suggest the involvement of TNF in hybridoma-induced killing.

A serine proteinase, which could not be localized to either the effector or the target, was detected as a 16 kDa DFP-binding activity induced during the lytic cycle. Involvement of a proteinase was also suggested by inhibition of lysis using the inhibitor DCI.

These results confirm that lysis can occur in the absence of perforin and the CCPs. Furthermore, they provide insight into a novel mechanism of lysis. The specific membrane contact between effector and target invokes a large number of metabolic changes which culminate in the death of the target cell. Although these events probably occur within the target, they can potentially take place in both cells. A model of lysis is presented in the following section. Further experiments are required to fully characterize the lytic mechanism employed by the cytolytic hybridomas.

Perforin alone has the capability to induce lysis (Duke *et al.*, 1989). However, cell death in this instance is not characterized by the fragmentation of target cell DNA into an oligonucleosomal ladder (Shi *et al.*, 1992b), seen during both NK and CTL-mediated lysis (Duke *et al.*, 1983; Russell, 1983) as well as during apoptosis (Wyllie *et al.*, 1980). However, unlike apoptosis, CTL-mediated lysis does not require protein synthesis (Duke *et al.*, 1983). This discrepancy between perforin-

mediated lysis and the observations with CTL suggests that other molecules must be involved. The discovery that the CCPs have the capability to mediate DNA damage suggests that they may also be required to facilitate target cell death (Munger *et al.*, 1988; Hayes *et al.*, 1989; Shi *et al.*, 1992a). These observations have not resolved the controversy of whether or not fragmentation is required for target cell death or is simply a consequence of lysis.

One of the major problems in examining the role of DNA fragmentation in target cell death is the inability to separate osmotic lysis, mediated primarily by perforin, from apoptotic cell death induced by a combination of perforin and other mediators. Several systems have been developed to examine the killer cell independent lysis (KCIL) phase of the lytic cycle (Martz, 1975; Golstein and Smith, 1976; Gately and Martz, 1977; Hiserodt *et al.*, 1982a). However, the addition of EDTA, EGTA, and/or dextran made it impossible to evaluate the subsequent fate of the target cell.

To overcome these problems, the "programming for lysis" assay was developed. This system takes advantage of an adherent CTL population which demonstrates high levels of cytolytic activity following brief stimulation with αCD3 mAb (Leo *et al.*, 1987). Targets were programmed during defined periods of incubation with the CTL. This step involved exocytosis and could be partially inhibited by the immunosuppressive agent CsA. Nonadherent cells (targets) were removed and several properties of death were examined.

Programming for as little as 15 minutes resulted in significant levels of DNA fragmentation with little associated chromium release. Viability determinations one day later indicated that target cell death correlated closely with the extent of DNA damage. Longer programming times resulted in greater osmotic lysis and the correlation between fragmentation and cell viability became more difficult to assess.

The role of DNA fragmentation in CTL-mediated lysis was examined using the endonuclease inhibitor ATA which inhibits apoptosis (Shi *et al.*, 1990) and NK-mediated lysis (McConkey *et al.*, 1990). ATA reduced the chromium release and DNA fragmentation induced by a number of

different types of effector cells. Control experiments confirmed no affect on CTL-target interactions or the resulting signal transduction. Although the activity of purified perforin was inhibited, this was not sufficient to explain all the results. When ATA was utilized in the programming assay, not only did it prevent DNA fragmentation, but it also significantly increased target cell viability. This observation suggested that DNA fragmentation is a cause, and not a consequence, of target cell death.

There is ample evidence to suggest that DNA damage is not always necessary to achieve death (Ratner and Clark, 1991; Ucker *et al.*, 1992). Instead, DNA fragmentation provides an alternate mechanism for killing cells which may be important when the effector to target ratio is low (as predicted *in vivo*) (Howell and Martz, 1989) or if perforin levels are too low to induce osmolysis.

The role of the CCPs in the lytic process has remained elusive until recently. Several functions have been postulated: activation of perforin (Hudig *et al.*, 1991); involvement in a cascade similar to the complement cascade (Gershenfeld *et al.*, 1988a); and detachment of effector from target (Tschopp and Nabholz, 1987). The observation that purified serine proteinases mediate DNA fragmentation after gaining access to the cell finally shed some light on the mystery (Munger *et al.*, 1988; Shi *et al.*, 1992b).

Three different inhibitors were utilized to examine the role of the proteinases in target cell lysis. A competitive substrate inhibitor of trypsin-like enzymes (BAEE) did not affect lysis or DNA fragmentation suggesting that these proteins were not involved. Two serine proteinases with trypsin-like specificities induce DNA fragmentation several hours after contact with the cell (Shi *et al.*, 1992b). Inhibition of these tryptases might not be detected during the short time course of the assays used. The inhibitor of chymotrysin-like enzymes, ATEE, prevented both lysis and DNA damage, with the greatest effect on fragmentation. Thus the major role of the chymases may be initiation of DNA fragmentation.

The general serine proteinase inhibitor DCI was the most effective agent (Harper *et al.*, 1985). It blocked both fragmentation and lysis at considerably lower levels than the other two. Lower doses produced a more pronounced inhibition of chromium release suggesting that the proteinase(s) involved in membrane damage were different from, and more sensitive to DCI than, the proteinase(s) involved in DNA fragmentation. More importantly, the effect of DCI on fragmentation was observed only if it was added at the beginning of the assay. DCI was incapable of blocking DNA damage when added following as little as 15 minutes of programming. However, it could still inhibit chromium release. These results further suggest a dual role for the proteinases: a very early role in initiating the events involved in DNA fragmentation and a continuing role in mediating the membrane damage which leads to target cell lysis.

Two discrete lytic mechanisms can be proposed based upon previous research and the results presented in this thesis. These pathways are presented in Figure 7.1. Granule-containing CTL and NK are represented by the pathway on the left. Contact between the CTL (or NK) and its target results in exocytosis of perforin and the CCPs. Perforin channels formed in the target cell membrane can have two functions. If enough perforin is present, osmotic lysis of the target will result (not shown). This most likely occurs at the higher effector to target ratios utilized during *in vitro* killing assays. When the E:T ratio is low so that perforin levels are sublytic, osmotic lysis does not occur. Instead, the CCPs enter the target via the perforin channels and play a role in two processes: initiation of DNA fragmentation, possibly by activating the endonuclease involved, and membrane damage. These processes lead to target cell death with the characteristics of apoptosis.

Agranular CTL, such as *in vivo* PEL and the cytolytic hybridomas, utilize a different lytic mechanism (outlined on the right side of Figure 7.1). Specific receptor-mediated contact between effector and target transmits an unidentified death signal to the target cell. This message results in considerable metabolic activity including protein

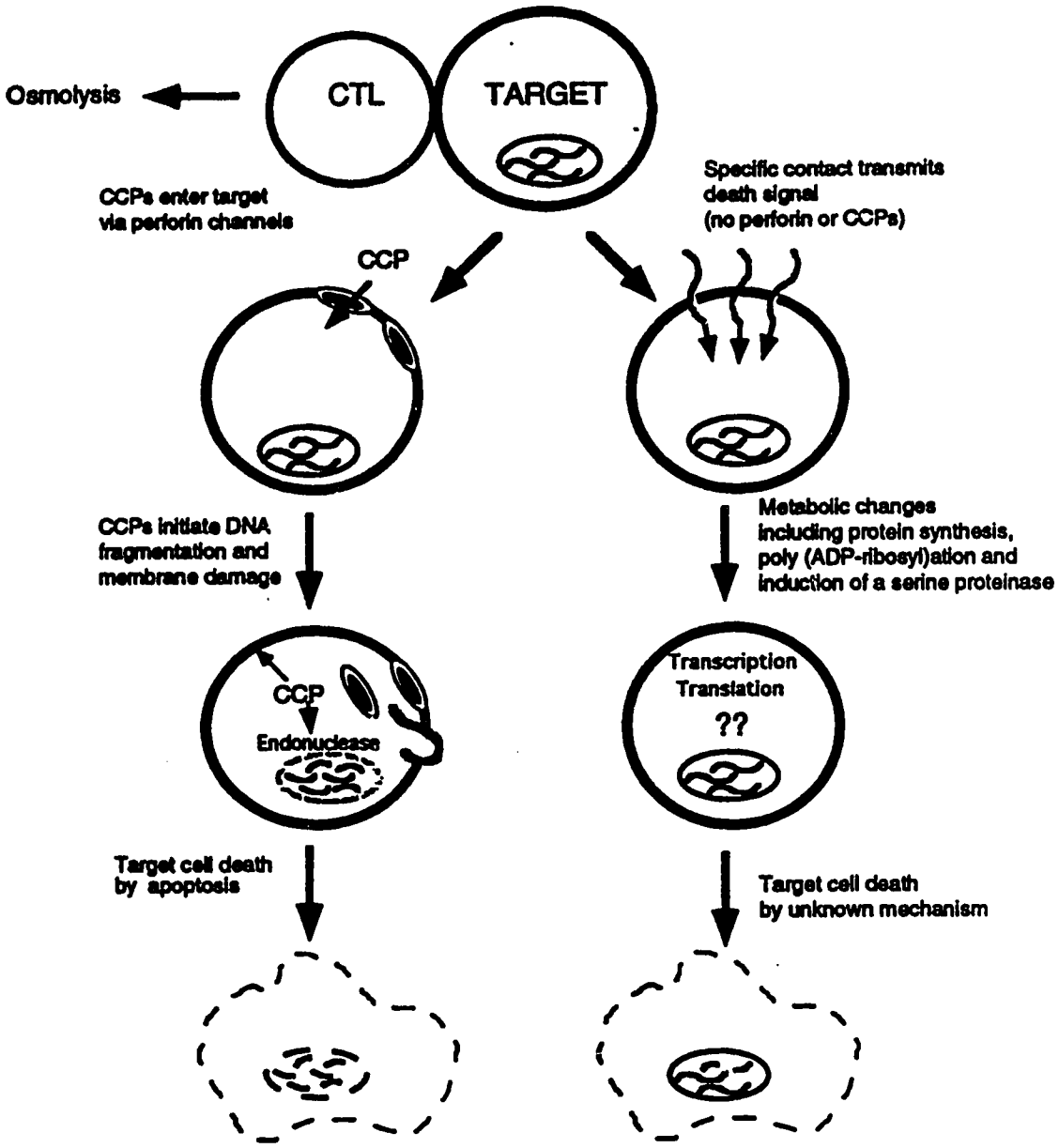


Figure 7.1: Proposed lytic mechanisms of the cellular immune system. Results presented in this thesis suggest that there are two lytic pathways: one used by CTL containing perforin and the CCPs (left) and one used by CTL which express neither (right).

synthesis, poly(ADP-ribosyl)ation, and induction of a serine proteinase. The consequence of these events is target cell lysis with little associated DNA fragmentation.

The results presented in this thesis support the hypothesis of alternate lytic mechanisms. CTL containing granules lyse targets by a mechanism involving the transfer of CCPs which mediate both DNA fragmentation and loss of membrane integrity. In targets susceptible to DNA fragmentation, this damage can be a cause of death. In contrast, CTL without granules have developed a mechanism with unique characteristics to lyse their targets. Whether this pathway is also utilized by granular CTL under certain circumstances remains to be determined. Regardless, the existence of several discrete mechanisms ensures that CTL have the capability to defend the body against all types of pathogens and oncogenic cells. If the target is not susceptible to one mechanism, it might be to another. Thus, the cellular immune system is equipped to deal with all possible problems!

FUTURE DIRECTIONS

The observation that the hybridomas employ a novel cytolytic mechanism poses several challenges. For example, what are the proteins synthesized during the lytic cycle and what is their function? What is the identity of the induced DFP-binding activity? Is it a novel serine proteinase? What role does it play in the death of the target cell?

It might be possible to address some of these questions by labelling proteins with ^{35}S-methionine during the lytic cycle. Separation of the proteins by SDS-PAGE followed by autoradiography should indicate the sizes of those synthesized during the short labelling period. Standard protein isolation techniques could be employed to purify these proteins, as well as the ^{3}H-DFP binding protein. In addition antibodies could be raised against each to help in their isolation and in the determination of their functions. Alternatively, differential screening of cDNA libraries may be useful to identify genes specific to the cytolytic hybridomas, as well as to freshly isolated PEL cells.

Several other questions also arise. What are the nature of the ion fluxes in the target cell? Are there changes in membrane fluidity? The technology exists to address these questions. It will be interesting to determine if granule-containing CTL also have the capacity to use this mechanism. That is, does it represent a novel pathway or is it simply an alternate mechanism utilized to increase killing efficiency under different physiological conditions? These questions await identification of the effector molecules.

The programming for lysis assay provided a useful assay for examining a number of characteristics related to target cell death. More importantly, this assay is potentially useful for examining the transfer of CTL-specific proteins (i.e., perforin and the CCPs) to the target during programming. CTL 21.9(IA) could be labelled with ^{35}S-Met and then incubated with target cells. Preparation of target cell lysates, followed by SDS-PAGE and autoradiography, should reveal the presence of labelled CTL proteins within the target.

The observation that DNA fragmentation causes target cell death was a significant first step. Isolation of the endonuclease involved in target cell DNA damage permits development of specific inhibitors. These can be used to further clarify the role of DNA degradation in both target cell death and apoptosis.

It is also important to determine the extent of similarity between apoptosis and CTL-mediated target cell death. For example, are the types of proteins involved in controlling apoptosis similar in function or nature to the CTL proteins which induce target cell apoptosis? In order to address this question it is necessary to understand the mechanisms which are responsible for mediating DNA fragmentation. Numerous labs are currently engaged in this type of research.

The proteinases, each with a unique substrate specificity, are expressed at various times in response to different stimuli (Prendergast *et al.*, 1992). This observation is significant, especially when it has also been observed that target cells are differentially sensitive to the effects of each of these proteins (Shi *et al.*, 1992a). The task now is to identify the biological substrates for each of the CCPs. This may

help to clarify the role of the proteinases in both the lytic process, and in the initiation of DNA fragmentation. Each of the seven currently known mouse proteinases offers a unique challenge!

In conclusion, it has been only 7-8 years since the identification of perforin and the cytotoxic cell proteinases. Significant progress has been made toward understanding the mechanisms by which CTL destroy their targets. However, considerable effort is required to achieve the level of understanding necessary to specifically manipulate CTL so that they will destroy selected targets in a designated fashion. It is certainly a goal worth working toward!

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