Biochemistry

 Volume 27, Number 8 April 19, 1988

Perspectives in Biochemistry

Cytotoxic T Lymphocyte Mediated Cytolysis

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Received October 20, 1987; Revised Manuscript Received December 17, 1987

A cytolytic effector cell of the immune system recognizes and lyses cells carrying non-self-epitopes, such as virus-infected cells, tumor cells, or grafted tissues [for reviews, see Berke (1985), Herberman et al. (1986), and Henkart (1985)]. For example, cytolytic T lymphocytes (CTL) that are generated as a result of viral infection can specifically destroy virus-infected cells. In spite of intensive research, we still do not understand how CTL irreversibly damage target cells, and only in the last few years have studies aimed at clarifying this issue entered a molecular stage of investigation.

The CTL-mediated lysis of a specific target can be conveniently divided into three phases (see Figure 1):

(*1*) *Recognition and Binding.* CTL are thought to adhere to their target cells via a number of cell surface molecules. The initial contact is made nonspecifically by two CTL–cell adhesion molecules found on the surface of lymphocytes, i.e., LFA-1 (lymphocyte function associated protein 1), a heterodimer consisting of an α subunit of 180 kDa and a noncovalently linked β subunit of 95 kDa, and CD-2 (cluster of differentiation), a 45–50 kDa glycoprotein. These molecules interact with the two target cell surface proteins called ICAM (intercellular adhesion molecule) and LFA-3, respectively. ICAM is molecularly still poorly defined. LFA-3 has a molecular mass of 55–70 kDa and displays a wide tissue distribution. This Mg^{2+}-dependent interaction is strengthened by interactions between the T cell receptor complex (T_i–T3) on the CTL and a combination of its cognate antigen and major histocompatibility complex (MHC) coded molecules on the target cell. The T cell receptor consists of the T_i α/β heterodimer, which carries the clonal specificity through its variable regions, and the associated CD3 complex. The avidity of these cell–cell interactions is enhanced further by the binding of CD8 (mouse Lyt-2), a membrane protein of the immunoglobulin superfamily, to monomorphic determinants of the appropriate MHC molecule on the target cell [for reviews, see Springer et al. (1987), Littman (1987), Allison and Lanier (1987)].

(*2*) *"Lethal Hit" Delivery.* Occupation of the T_i–T3 complex of the CTL causes a local Ca^{2+} influx (Weiss et al., 1986; Poenie et al., 1987), which acts as a signal for the so-called "lethal hit" delivery phase. This is a Ca^{2+}-dependent process that leads to a drastic morphological reorganization of the CTL and the subsequent delivery of molecules "lethal" to the target cell. The present review will focus mainly on this killing event. Once the lethal proteins have been released from the CTL, the continued presence of the CTL is not required for target cell death; i.e., the CTL may detach, "recycle", and go on to lyse other target cells.

(*3*) *Target Cell Lysis.* Several dramatic changes are observed in the target cell during the lytic phase. Massive Ca^{2+} influx and changes in the overall shape of the target cell are followed by dilatation of the endoplasmic reticulum and chromatin condensation (Duvall & Wyllie, 1986). These morphologic changes are accompanied by DNA fragmentation (Russell et al., 1980; Duke et al., 1983) and, ultimately, because of cytoplasmic membrane damage, the release of macromolecules (and ^{51}Cr, which is used to assay for this event) into the extracellular environment. The dual disintegration of DNA and plasma membrane is clearly different from the events observed in complement-mediated lysis, during which DNA fragmentation is not observed. However, as in complement-mediated cytolysis, membrane debris recovered from the target cells carries morphologically distinct proteinaceous circular lesions (Dourmashkin et al., 1980; Podack & Dennert, 1983).

Several molecules of CTL origin are presumed responsible for delivery of the lethal hit to the target cell (second phase). In order to avoid undue speculation, however, only proteins that have been cloned and/or purified will be discussed here; these include the pore-forming protein perforin/cytolysin, a family of proteases that we have called granzymes, and lymphotoxin [tumor necrosis factor β (TNF-β)].

Cytoplasmic Granules of CTL Are Cytotoxic, and Their Content Is Released upon Target Cell Contact

The pore-forming protein perforin/cytolysin and the "granzyme" proteases are candidates suspected of being cy-

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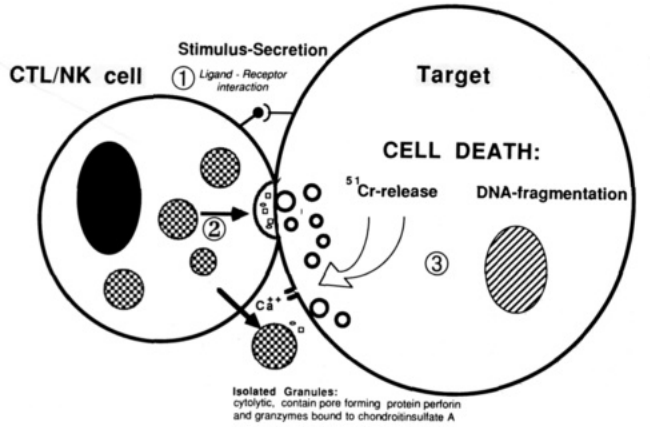

FIGURE 1: Schematic model of CTL-mediated cytolysis. CTL–target cell interaction is initiated by a specific Mg^{2+}-dependent adhesion step (1), followed by a Ca^{2+}- and temperature-dependent delivery of lethal proteins (2). TNF-β, the pore-forming protein perforin/cytolysin, and the granzyme proteases are possible candidates for these lytic proteins. After attack by the CTL, the target cell sustains extensive membrane damage, leading to the release of macromolecules and ^{51}Cr, and its DNA becomes degraded (3).

toloytic factors of the CTL. These proteins reside in numerous dense cytoplasmic granules of CTLs and are stored bound to chondroitin A proteoglycan (Schmidt et al., 1985). The observation that isolated granules are cytolytic to various tumor cell lines and red blood cells (Podack & Königsberg, 1984; Henkart et al., 1984) without specificity indicates the importance of these granule-associated molecules in cytolysis. Moreover, there is also considerable evidence from morphologic and functional analyses that the granular components are released upon CTL–target cell conjugation. After initial target cell recognition and triggering of the T cell receptor complex, the CTL undergoes a transient rise in intracellular Ca^{2+} concentration (Poenie et al., 1987), followed by an extensive cytoplasmic rearrangement. The microtubule organizing center, the Golgi apparatus, and the cytoplasmic granules are polarized toward the site of cell–cell contact (Zaguri, 1982; Kupfer et al., 1986; Yanelli et al., 1986). These events are followed by granule exocytosis and release of granular components into the intercellular space, where they form a diffusion-limited microenvironment with a high local concentration of lytic molecules. Proteases and proteoglycan molecules have been detected in the supernatants of CTL–target cell conjugates (Schmidt et al., 1986; Young et al., 1986a; Pasternack et al., 1986; Garcia-Sanz, 1987), supporting a stimulus–secretion model. The proteins are initially sequestered and are then secreted, allowing them to exert their lytic action on the target cell.

Perforin

The protein that produces transmembrane lesions, as observed on the target cell membrane after granule- or CTL-mediated cytolysis, has been isolated and characterized from CTL, NK cell lines, and large granular lymphocytes (Masson & Tschopp, 1985; Podack et al., 1985; Young et al., 1986b; Zalman et al., 1986). Perforin (also called cytolysin, pore-forming protein, or C9-related protein) is a hydrophilic, single-chain protein with a molecular mass of 66–75 kDa. In the presence of Ca^{2+} (the lethal hit phase of CTL–target cell interaction is Ca^{2+} dependent), perforin undergoes dramatic ultrastructural changes involving a transition from its monomeric, hydrophilic state into an amphiphilic, polymeric form. The tubules formed by polymerized perforin (polyperforin) have an inner diameter of 16 nm (Figure 2) and a height of 16 nm. Each tubule contains approximately 12–18 protomers.

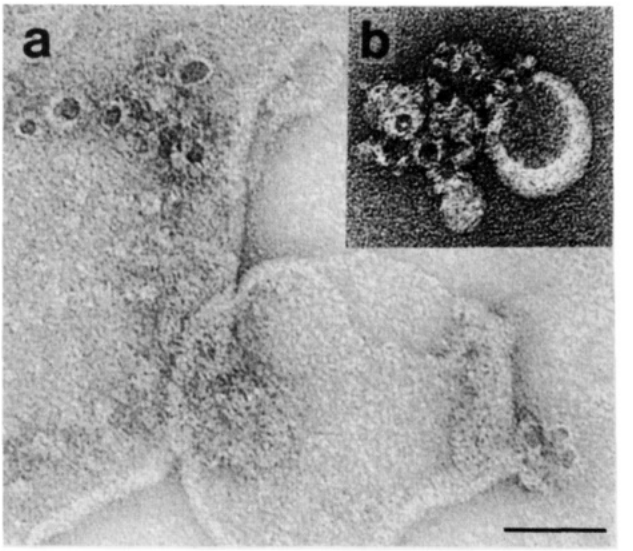

FIGURE 2: Circular lesions on target cell membranes. (a) Sheep red blood cells lysed by purified mouse perforin in the presence of Ca^{2+}. Lesions of up to 16-nm inner diameter are observed. (b) Purified human complement component C9, added to egg lecithin vesicles. In contrast to natural membranes, artificial vesicles are lysed in the absence of complement components C5b through C8. Only the presence of Zn^{2+} is required. The inner diameter of the tubule is 10 nm.

In the presence of biological or artificial membranes, the nascent amphiphilic form of perforin binds to and inserts into the target membrane, forming large transmembrane channels. Pores formed by perforin in the plasma membrane are large enough to permit the exchange of ions, which ultimately results in cell death. As demonstrated with whole granules, the purified perforin also lyses a variety of cells without specificity. The fact that perforin is rapidly inactivated by lipoproteins (Tschopp et al., 1986a) may explain why localized secretion of perforin does not result in innocent bystander lysis.

Pore formation by perforin is in many respects similar to the channel formation induced by the ninth component of complement (Figure 2; Tschopp et al., 1982) or other pore-forming lytic proteins like bacterial and amoebal toxins (Bhakdi & Tranum-Jensen, 1983; Young et al., 1982). The demonstration that perforin and C9 possess common antigenic epitopes (Tschopp et al., 1986b; Young et al., 1986c) has been confirmed by analysis of the primary structure of perforin (Lowrey et al., 1987). The structure of C9 and its mode of lipid insertion and channel formation have been well studied [for a review, see Podack and Tschopp (1984)]. Monomeric C9 binds to a preassembled C5b–C6–C7–C8 complex on the complement-activating target cell. Binding of C9 occurs at 4 °C, but only at 37 °C does the C9 molecule rearrange from a globular 8-nm hydrophilic monomer into an amphiphilic extended form of 16 nm in length (Figure 3). This conformational transition in C9 simultaneously expresses the hydrophobic surface originally buried in the globular protein. C9 inserts into the membrane by means of this hydrophobic domain and then binds to and induces unfolding of additional C9 molecules. After 12–18 C9 molecules have polymerized in a circular array [poly(C9)], ring closure occurs, forming a tubule of remarkable stability (resistant to dissociation by sodium dodecyl sulfate). It is important to point out that the closed ring structure is not essential for transmembrane channel formation. C9 monomers and oligomers are capable of formation of smaller diameter channels, by repelling the lipid bilayer (Bhakdi & Tranum-Jensen, 1986; Dankert & Esser, 1985; Ramm et al., 1982).

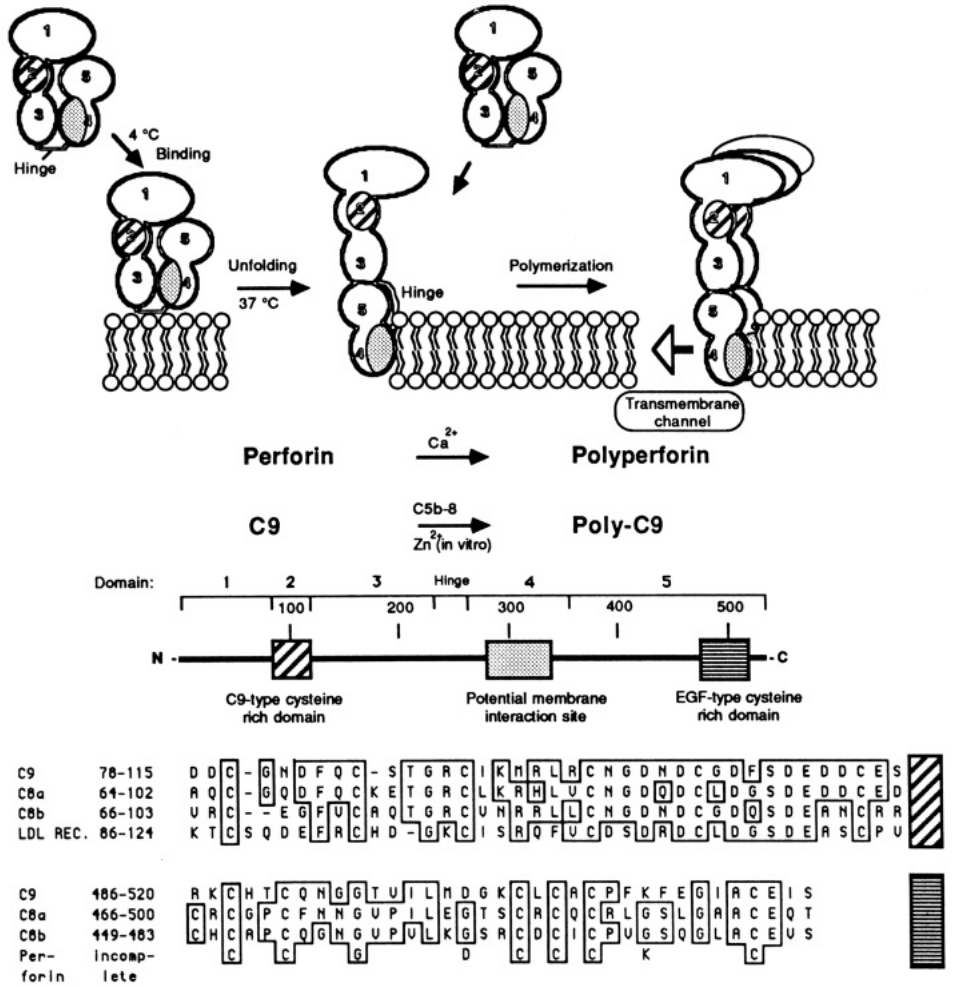

FIGURE 3: Speculative model of perforin and C9 polymerization and lipid insertion. The initially hydrophilic monomeric proteins bind to the target membrane (C5b-8 on the cell surface is essential for C9 binding in vivo; in vitro Zn^{2+} can replace C5b-8; Ca^{2+} is a cofactor for perforin). Subsequent to binding, a drastic structural rearrangement occurs, leading to polymerization and a transition to amphiphilic molecules. The hinge region of C9 and perforin "unfolds" during this transition, which, however, does not entail a dramatic change in the secondary structure of C9, in agreement with the small changes observed in the circular dichroic spectra (Tschopp et al., 1982). Since the membrane binding site within poly(C9) is present on the tip of the cylindrical pore, one has to postulate that, during the rearrangement of the five C9 domains, the lipid interacting domain (domain 4) has to move to the end of the protein. Subsequently, "unfolded" C9 (perforin) binds to an additional molecule of monomeric, hydrophilic C9 (perforin) and induces its unfolding, resulting in C9 (perforin) polymerization. Transmembrane channels are formed by the repulsion of the lipid molecules of the membrane. The structural organization and the domain structure of complement components C9, C8α, and C8β and (most likely) perforin molecules are shown below. The amino acid sequences of the two cysteine-rich domains of C9 are compared.

In contrast to perforin, the functionally important domains within C9 have been partly defined. Analysis of the primary structure (Stanley et al., 1985; DiScipio et al., 1984) reveals that C9 contains two distinct cysteine-rich domains and a region where amphipathic α-helices, which could be involved in lipid binding, can be predicted from the primary structure (Stanley & Herz, 1987; Figure 3). A membrane-restricted photoactivated label binds to a segment of C9 extending from amino acids 245 to 346 (Schäfer et al., 1987). This observation indicates that the membrane interaction site in C9 is localized to a region defined by these 102 residues.

Unfolding and polymerization of C9 (and perforin) may involve the mechanism schematically shown in Figure 3. Monomeric C9 contains a cryptic domain (domain 2 in the model) that is highly homologous to an LDL–receptor domain known to interact with apoprotein B or E (Stanley & Herz, 1987; Yamamoto et al., 1984). Upon unfolding of the monomeric C9 molecule, this "C9-type/LDL-receptor" cysteine-rich domain is exposed, and it is this domain that is at least partly responsible for binding and induction of unfolding of subsequent C9 molecules. It seems that an LDL receptor–apolipoprotein-like interaction is important in intermolecular C9–C9 interaction. Interestingly, not only does a peptide corresponding to the segment shared by the LDL receptor and C9 influence C9 activity (Tschopp et al., 1986b) but also all inhibitors known to interfere with LDL-receptor/LDL interactions abrogate C9 and perforin activity (Tschopp & Masson, 1987).

The same basic domain structure found in C9 is found in C8α and C8β (Häfliger et al., 1987; Howard et al., 1987; Rao et al., 1987) and in perforin, although the complete amino acid sequence of perforin is still unknown. This common architecture would suggest it is the arrangement of structural elements as discussed above that plays a significant role in function. Consequently, proteins involved in lysis of cells, either by the complement system or by CTL, may undergo similar hydrophilic–amphiphilic transitions.

GRANZYMES

Protease inhibitors suppress the lytic activity of CTL, indicating that proteases may be involved in CTL-mediated killing (Chang & Eisen, 1980; Hudig et al., 1984). CTL lines contain much higher levels of serine esterase activity (based on a trypsin-sensitive substrate) than noncytolytic cells

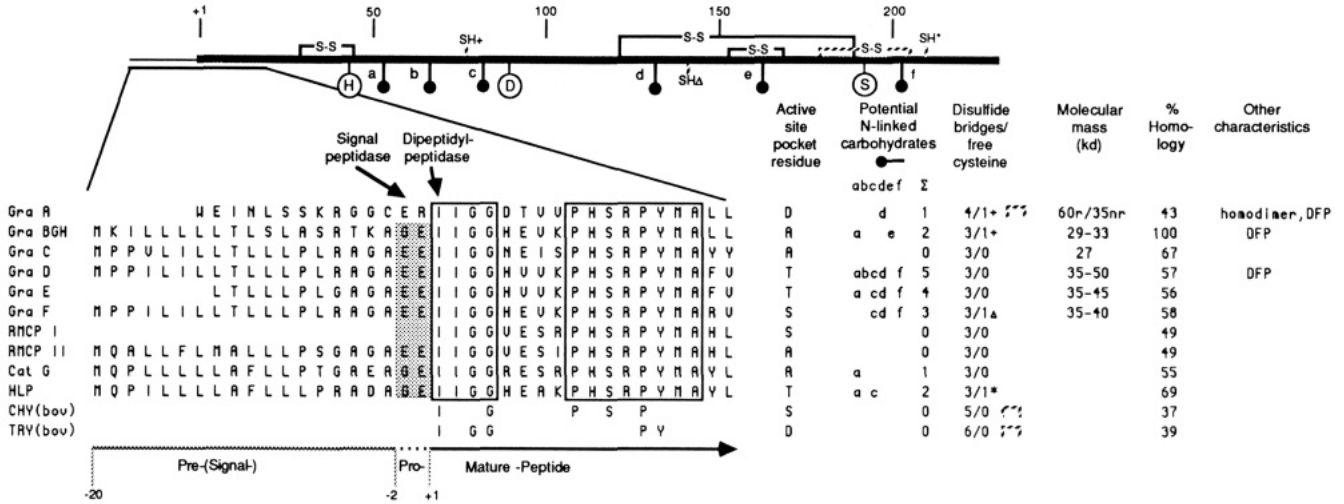

FIGURE 4: Structural organization of the granzyme protease family. Proteases (granzymes) of granules of mouse cytotoxic T lymphocytes characteristically contain three disulfide bridges and the His, Asp, Ser triad known to form the catalytic center of serine esterases. Some granzymes are highly glycosylated. Amino acid sequences are highly conserved between positions 1–4 and positions 9–16. Granzymes are synthesized as prepromolecules containing a signal peptide (amino acid residues −20 to −3) and an acidic activation dipeptide (−2 and −1). Proteases with high sequence homology to granzymes are found in the granules of rat mast cells (rat mast cell proteases I and II), in human neutrophils (cathepsin G), and in human T cells (human lymphocyte protease, HLP). Other names for granzyme A include CTLA-3, H-factor, TSP-1, and SE-1; granzyme B has also been called CTLA-1, CCP1, and SE-2.

(Pasternack & Eisen, 1985). Indeed, the majority of the proteins (approximately 85%, versus 10% for perforin in mouse CTL lines) present in the cytoplasmic granules belong to a family of serine proteases, designated granzymes A–H (Masson & Tschopp, 1987). At the time of this writing, all eight granzymes have been characterized biochemically, and cDNA clones encoding six of them have been isolated (Lobe et al., 1986; Gershenfeld & Weissman, 1986; Brunet et al., 1986; Jenne et al., 1988). Granzymes are all highly homologous to each other (at least 43%) and contain the His, Asp, Ser triad known to form the catalytic center of serine esterases (Figure 4). The mature proteases all begin with the common N-terminal sequence Ile-Ile-Gly-Gly, having variations at residue positions 5–8, with residues 9–16 identical. Granzymes D, E, and F are highly glycosylated proteins. Interestingly, three other non-CTL proteases stored in cytoplasmic granules, rat mast cell proteases I and II (RMCP I and II) and cathepsin G of neutrophils, contain these same conserved sequences (Woodbary et al., 1978; Benfey et al., 1987; Salveson et al., 1987). Human lymphocyte protease (HLP) is the fourth protease currently known to belong to the granzyme family. The existence of this protease was predicted from the nucleotide sequence of a human T cell specific cDNA clone (Schmid & Weissmann, 1987) and may well represent the human homologue of one of the mouse granzymes. Other characteristics of the granzyme family are the basic nature of the polypeptide chain and the absence of a particular disulfide bridge normally present in chymotrypsin or trypsin. Granzyme A is an exception, because in addition to having four disulfide bridges, it occurs as a disulfide-linked homodimer (Pasternack et al., 1986; Masson et al., 1986; Simon et al., 1986a). Moreover, the degree of overall homology between granzyme A and the granzymes B–H, RMPC I and II, cathepsin G and HLP is relatively low (Figure 4). Granzyme A has a trypsin-like activity, as indicated by the aspartic acid residue in the binding pocket and by its substrate specificity for arginine–X bonds. Good substrates have not yet been found for the other granzymes even though Ala, Thr, or Ser is found in the binding pocket, suggesting a chymotrypsin-like specificity. Except for granzyme A, all granzymes contain the acidic dipeptide Gly-Glu or Glu-Glu (propeptide) following the signal peptide (prepeptide). After signal peptide removal, the acidic N-terminal dipeptide of granzymes B–H must be cleaved to yield the mature protease. We do not know where in the cell this processing occurs, but the cytoplasmic granules are likely candidates. Since granzymes are neutral proteases, they will only exhibit activity upon exocytosis.

The function of the granzymes is poorly understood. The direct involvement in the cytolytic process that had been initially proposed has not yet been confirmed. Unlike perforin, purified granzymes alone have no cytolytic activity. Further, phenylmethanesulfonyl fluoride (PMSF) treatment does not affect the lytic activity of granules (Masson et al., 1986). CTL's whose granzyme A has been inactivated lose only a small portion of their activity (Henkart et al., 1987). This observation does not exclude a role for granzymes B–H in CTL-mediated cytotoxicity, since PMSF may mediate inactivation of only granzyme A. There is no evidence that granzymes act in a proteolytic cascade leading to the activation of perforin, as is the case in the complement cascade system. Granzyme A is present not only in $CD8^+(Lyt2^+)$ cytolytic lymphocytes but also in the noncytolytc $CD4^+(L3T4^+)$ helper lymphocyte population (Simon et al., 1986b; Garcia-Sanz et al., 1987). In addition to CTL, granzyme B appears to be also expressed in bone marrow derived mast cells (Brunet et al., 1987). Although the cellular distribution of granzymes C–H has not been studied, it appears that the granzymes are not unique to CTL. Thus, the role of granzymes in the cytolytic event may only be indirect. It has been suggested that granzymes may be important in facilitating detachment of CTL from the target cell. It is well-known that CTL, after conjugate formation and the delivery of the toxic molecules, can detach and "recycle", i.e., continue and lyse additional targets. The dissociation of effector and target cells may be mediated by granzymes, which would act on the various receptor and ligand molecules involved in CTL–target cell recognition and conjugate formation. For granzyme A, it has also been shown that it cleaves extracellular matrix proteins (Simon et al., 1986b). Granzyme A may therefore allow stimulated T cells to invade and penetrate vessel walls, thereby facilitating their migration. Yet another role for granzyme A suggested by Simon et al. (1986a) is its involvement in B cell growth. Earlier studies have shown that thrombin and trypsin can drive B cells into proliferation. This protease-in-

duced B cell growth can also be achieved by granzyme A.

LYMPHOTOXIN

Lymphotoxin was originally described as a cytotoxic activity secreted by activated T cells (Granger & Williams, 1968; Ruddle & Waksman, 1968). Its role in the cytolytic process has remained controversial. The purification of lymphotoxin secreted by a human lymphoblastoid cell line (Aggarwal et al., 1985b) and the subsequent cloning of its cDNA and gene (Gray et al., 1984; Nedwin et al., 1985) have helped to clarify the situation. Lymphotoxin was shown to be a close relative of the macrophage cytotoxic factor, tumor necrosis factor (TNF), and to have biologic activities indistinguishable from those of TNF, probably because the two proteins share the same receptor (Aggarwal et al., 1985a). Therefore, they are now called TNF-α (formerly TNF) and TNF-β (formerly lymphotoxin). The genes for the two TNF's are closely linked in mouse, man, and rabbit (Nedospasov et al., 1986a,b, and unpublished results).

TNF-β is a glycoprotein with a monomeric molecular mass of 25 kDa (18.5 kDa without glycosylation). The sequence of TNF-β cDNA reveals that the primary translation product contains a signal peptide of 33 amino acids (Gray et al., 1984). Activated CTL secrete TNF-β continuously, making it unlikely that the protein accumulates in cytolytic granules. The proposed role of TNF-β in CTL-mediated cytolysis is based on the fact that many CTL lines secrete TNF-β when they are incubated with cognate target cells and that TNF-β-containing cell supernatants induce limited DNA degradation in some tumor cell lines [for a review, see Ruddle and Schmid (1987)].

However, several lines of evidence argue against a direct role for TNF-β in target cell killing by CTL. First, it is known that many cell lines that can be killed by CTL are refractory to the toxic effects of TNF-β (Sugarman et al., 1985). Second, the appearance of TNF-β mRNA after antigenic stimulation and secretion of the protein takes several hours (C. B. Wilson, personal communication; our unpublished results), and CTL kill their targets within minutes of contact. Third, we have identified several CTL lines that can kill their targets without ever producing detectable quantities of TNF-β mRNA or protein. It seems much more likely that TNF-β is a member of the growing family of lymphokines that are instrumental in the generation of an inflammatory response [see Old (1987)]. The toxic effects of the protein, which are limited to certain tumor cell lines, may simply reflect an alternative mechanism for elimination of some types of metastatic growths.

CONCLUSION

The evidence that stimulus-secreted perforin is involved in target cell lysis by CTL is considerable. Purified perforin is lytic and forms polyperforin channels deposited on CTL-lysed target membranes. Thus, CTL and most likely perforin-containing NK cells lyse their targets, at least in part, by forming large transmembrane channels that disrupt the membrane integrity of the target cell. This lytic mechanism has proved its efficacy in the complement system and in some bacterial and amoebal toxins.

It seems unlikely, however, that perforin is the sole mediator of the CTL lethal hit, because in contrast to complement-mediated lysis, DNA fragmentation is always associated with cell death mediated by CTL. Thus, it is possible that granzymes, TNF-β, or other yet unpurified molecules could play an important supporting role in the efficient delivery of a lethal hit, either synergistically with or independently from perforin. Indeed, there is increasing evidence that CTL may also be lytic in the absence of perforin. It has been recently shown for one particular target cell that lysis can occur in the absence of Ca^{2+} (Ostergaard et al., 1987; Trenn et al., 1987), a metal ion that is absolutely required for the exocytosis of granules and for perforin activity. Also, to study CTL-mediated cytotoxicity, most investigators have been utilizing Il-2-dependent, long-term cultured CTL lines, which always express granzyme A and perforin, whereas these two molecules seem to be lacking in highly cytotoxic, in vivo induced effector cells (Dennert et al., 1987). Thus, there is still a long way to go toward a full understanding of the mechanisms that lead to target cell lysis.

ACKNOWLEDGMENTS

We thank Drs. D. Jenne and M. Nabholz for helpful discussions and E. Burnier, Z. Freiwald, and R. Etges for help in preparing the manuscript.

Registry No. Protease, 9001-92-7.

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