Reviews

Perforin and its role in T lymphocyte-mediated cytolysis

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Abstract. The killing mediated by cytotoxic T lymphocytes (CTL) represents an important mechanism in the immune defence against tumors and virus infections. The lytic mechanism has been proposed to consist of a polarized secretion of granule-stored molecules, occurring on effector-target cell contact. By electron microscopy, membrane deposited, pore-like lesions are detected on the target cell membrane during cytolysis by CTL. These structures resembled strikingly pores formed during complement attack.

Granules of CTL isolated by nitrogen cavitation and Percoll gradient centrifugation were shown to retain cytotoxic activity. Further purification of proteins stored in these granules led to the discovery of a membranolytic protein named perforin which was capable of polymerizing into pore-like structures. In addition to this cytolytic protein, a set of serine esterases was found as well as lysosomal enzymes and proteoglycans, whose function are not yet clearly defined. The role of perforin in the cytotoxic process is currently being explored by ablating the active gene in mice.

Key words. Cytotoxic T lymphocytes; granules; perforin; gene targetting.

Introduction

The essential function of the immune system is defence against foreign invaders such as bacteria, viruses or even allogeneic cells which are recognized as 'non-self'. Primitive animal forms possess *innate* or *non-specific* immune mechanisms, for example phagocytosis of bacteria by specialized cells. Additionally, vertebrates have evolved an *adaptive* or *acquired* immune response which provides a flexible, specific and more effective reaction to the different infections. The adaptive immune response is characterized by memory, specificity and the recognition of non-self.

When antigen enters the body, two different types of adaptive immunological responses may occur:

1) *The humoral response.* This consists of the synthesis and release of antibodies into the blood and other body fluids. Binding of a free antigen molecule to the specific B lymphocyte antigen-receptor (surface or membrane immunoglobulin, IgM) induces proliferation and differentiation of B lymphocytes into plasma cells which secrete large amounts of specific, soluble immunoglobulins (antibodies, often IgG, but also IgA, IgD, IgE, or soluble IgM). These antibodies then bind to the circulating antigens, thus allowing their elimination by other components of the immune system. The antibodies may also bind to cell-bound antigens, for example coating bacteria to enhance their phagocytosis. Cells which have cell surface-bound antibodies are susceptible to lytic attack by another component of the humoral immune system, i.e. the complement system which inserts a pore forming complex (the membrane attack complex, MAC), consisting of the five proteins C5b, C6, C7, C8, and C9, into the cell membrane (for reviews, see Bhakdi et al.[3] and Podack and Tschopp[47]).

2) *The cellular response.* This includes immunologically relevant cytocidal cells such as macrophages, cytolytic T lymphocytes (CTL) and natural killer (NK) cells, and provides the main defence against intracellular organisms and possibly also tumor cells. Upon activation, T lymphocytes mediate the cellular immune response by secreting soluble factors, known as lymphokines (the 'helper' function) and/or by killing target cells bearing the antigen. The function of T lymphocytes depends on close contact with other cells via molecules belonging to three different families of adhesion receptors, namely the Ig-superfamily, which includes the T cell antigen receptor (TCR), the integrin family, and the selectins (for reviews, see Davis and Bjorkman[10] and Springer[59]). T lymphocytes, in contrast to B lymphocytes, do not recognize soluble antigen, but bind to specific antigen only when it is present on the surface of cells in association with molecules of the major histocompatibility complex (MHC).

This review will focus on lymphocyte-mediated cell killing.

Lymphocyte-target cell conjugation

Lymphocyte-induced target cell lysis involves a complex series of events, one of the earliest undoubtedly being rapid specific binding of the CTL or NK cell to its target cell (TC), resulting in conjugate formation. Conjugation is a prerequisite for lysis. The conjugation event is mediated by the specific interaction of the TCR-CD3 complex with a combination of its cognate antigen and the class I MHC molecule and by the two pairs of accessory adhesion molecules, LFA-1 and CD2 on the CTL membrane, interacting with ICAM and LFA-3, respectively, on the TC membrane. The avidity of cell-cell binding is further enhanced by the CD8 component. Conjugation is dependent on the presence of Mg^{2+}, but not Ca^{2+}.

Post-conjugation cellular events

The signals transmitted to the effector cell following conjugation are not yet completely understood, but there is accumulating evidence suggesting that TCR-CD3 binding induces CTL membrane depolarization, calcium influxes, and phosphorylation of one of the CD3 polypeptide components[72]. These signals trigger the lytic machinery of CTL, and in particular, the delivery of the 'lethal hit', a Ca^{2+}- and temperature-dependent process. Conjugation is accompanied by the reorientation of their microtubule organizing center (MTOC) and centrioles towards the site of cell-cell contact[17,27,28]. Likewise, the Golgi complex becomes localized in the vicinity of contact regions. The MTOC/Golgi reorientation is not observed when CTL bind to TC in the presence of microtubule-disrupting agents[27].

Among the more prominent morphological features of CTL lines is the presence of numerous large, electron-dense cytoplasmic granules. The activation of resting lymphocytes is accompanied by the biogenesis of such granules[39] which are occasionally scattered through the cytoplasm or, more commonly, concentrated in the perinuclear region. Killer cell granules closely resemble vesicles of other cell types which are secreted via a regulated exocytotic pathway. Following conjugate formation, these granules are also polarized towards the cell-cell binding site[7,42,79] and have been observed by cinemicrography to fuse with the plasma membrane of CTL in the vicinity of the area of its contact with the TC[70].

The observations of the reorientation of cytoplasmic granules led Henkart and co-workers in 1982[19,20] to propose an attractive model for delivery of the lethal hit[22,44,58,65,71].

According to this model, the effector cell, upon appropriate effector-target cell conjugation, vectorially delivers dense cytoplasmic granules and their contents into the intercellular space at the site of contact between the target and effector cells. This process of degranulation releases, amongst other molecules, a pore-forming protein, perforin; a family of serine esterases, known as granzymes (seven members, A–G are known in mouse, only three, A, B, H have been reported in man), and proteoglycan molecules. These substances accumulate in the diffusion-limited microenvironment at high local concentrations. Once released from the granules, and in the presence of Ca^{2+}, perforin polymerizes on the TC membrane, forming transmembrane, ring-like pores, structurally similar to those caused by the complement membrane attack complex or bacterial toxins. The effector cell may then detach from the TC and recycle to lyse further targets, whilst lysis of the initial TC ensues.

It is well known that CTL, after having delivered the lethal hit to the TC, can detach and 'recycle' since their continued presence is not required[2,79]. CTL appear not to be inactivated as a result of deploying their lytic machinery, and can almost immediately participate in a new lytic interaction.

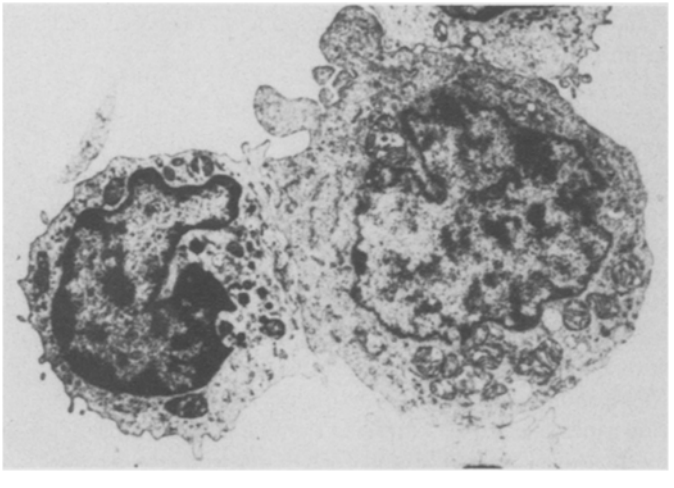

Figure 1. Electron micrograph of a CTL attacking a tumor target cell. The CTL, at the left, makes contact with the larger target cell. Electron dense granules are seen in the killer cell moving into the area of contact.

Membrane damage and nuclear DNA desintegration

Target cell lysis can be assessed by the release of radioactive molecules, the uptake of dyes which are excluded by intact viable cells, or by radiolabelling of residual cells. In 1968, Brunner et al.[6] modified the ^{51}Cr-release assay, long employed to measure complement-induced lysis of erythrocytes and nucleated cells, to measure lymphocyte-mediated cytolysis. This simple procedure has been used ever since with virtually no modification because of the excellent correlation between ^{51}Cr-release and cell lysis and the possibility of handling large numbers of samples. Since the ^{51}Cr-release assay measures the integrity of the TC membrane, it became apparent early on that membrane damage must occur at some stage during cell-mediated killing. Several observations then suggested that membrane damage might represent the primary site of cell injury. In 1974, Henney[24] used high molecular mass dextran markers to show that the initial T cell-induced membrane lesion was at least 9 nm in diameter, and proposed that water must have entered the TC during cytolysis, resulting in 'colloid osmotic' forces causing TC death. He also pointed out that his findings revealed a 'striking similarity to the nature of the antibody induced complement lesion in erythrocytes'. Later attempts at estimating the size of the 'holes' induced by lymphocytes mediating ADCC on resealed erythrocyte ghosts used macromolecules of known Stokes radius to estimate a maximal diameter of 15 nm for the pore[57]. Kinetic analyses of marker release revealed that the permeability increase of the TC membrane occurs concomitantly with the onset of the 'calcium-programming stage' for lysis[35].

In contrast to killing by osmotic lysis, a phenomenon called apoptosis occurs during cytotoxicity by both CTL and NK cells. This consists of the condensation of the

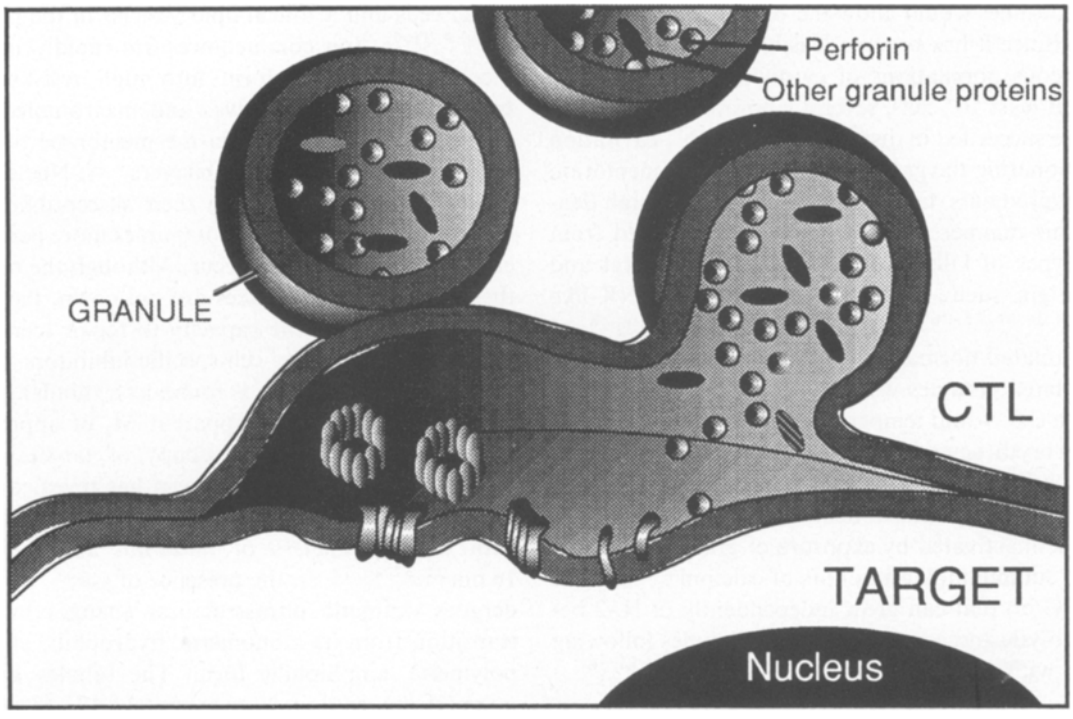

Figure 2. The hypothetical perforin/granule exocytosis model of lymphocyte-mediated cytotoxicity proposes that, upon effector-target cell conjugation, cytoplasmic granules, containing perforin, granzymes and proteoglycans, are exocytosed into the intercellular space at the contact site. In the presence of Ca^{2+}, perforin binds to the target cell membrane and finally polymerizes to form tubular, transmembrane lesions. These pores can then either directly, i.e. by disruption of ion channels, generate a signal that induces DNA fragmentation, or allow other granule-stored proteins, which act as messengers in cell death induction, to enter the target cell.

target cell chromatin and final cleavage of the DNA at the linker regions of the nucleosomes, producing a characteristic ladder pattern. This mechanism is thought to be induced by the effector cell, but the ultimate mediator(s) of DNA fragmentation is (are) probably derived from the target cell itself (see a more detailed discussion below).

This other, but not necessarily alternative, view of cell-mediated killing was initially suggested by studies which disputed the operational analogy between the cytolytic mechanisms mediated by complement and lymphocytes. Lymphocyte-induced damage of target cells, in contrast to complement attack or hypotonic shock, appears to be initiated from within the cell, in particular in the nucleus of the target cell, where rapid condensation of chromatin and nuclear membrane blebbing are the first signs of cell death [51]. Russell and co-workers, taking these events into account, have proposed the 'internal disintegration model' of lymphocytotoxicity (for reviews, see Russell [49] and Wyllie et al. [69]) and extended the initial observations by demonstrating early and rapid DNA breakdown in target cells lysed by CTL [50, 52]. In fact, within minutes of exposure to CTL or NK cells, but not to complement, the target cell DNA begins to be fragmented into nucleosome-sized fragment (180 bp or multiples thereof), which suggests the involvement of an endonuclease activity [13]. These events, which have been collectively referred to as apoptosis, are typical of cells undergoing so-called 'programmed cell death' during embryonic development [69], and of autoreactive cells in the thymus undergoing clonal deletion [34], or corticosteroid-induced death of thymocytes [8].

Perforin and the granule-exocytosis-model

Perforin, a granule-associated cytolytic pore-forming protein

The first structural evidence for pores on TC membranes was provided by Dourmashkin et al. [12] who examined erythrocyte ghost membranes after attack by lymphocytes in an ADCC reaction. Circular lesions with an internal diameter of ~ 15 nm were observed by negative staining. Podack and Dennert extended these findings to cloned NK cells [45] and H2-specific CTL [11] by describing two types of tubular lesions, one with an internal diameter of 16 nm, named polyperforin, and a second smaller lesion, with an internal diameter of 5 nm. The putative monomer that assembles these complement-like lesions on the TC membrane was named perforin. Young et al. [78] have reported the presence of similar lesions in vivo in acute idiopathic and viral myocarditis, diseases in which myocardial damage appears to be inflicted by cytolytic lymphocytes.

Isolation of hemolytic granules

If, in fact, cytoplasmic granules are involved in TC killing, then sub-cellular fractionation and isolation of

purified granules would allow the verification of this hypothesis. Since it has become feasible routinely to grow homogeneous populations of cytolytic effector cells to produce at least 10^9 cells, several laboratories, including ours, have succeeded in disrupting cells by N_2-cavitation and fractionating the granules by velocity sedimentation of Percoll gradients, taking advantage of their high density. In this manner, granules have been isolated from various types of killer lymphocytes of mouse, rat and human origin, such as LGL tumors[21], CTL or NK-like cell lines[9,26,46,75,76], CTL hybrids[36], LAK cells[23], or CD3-stimulated normal T lymphocytes[16,81,82].

These isolated granules were all shown to be highly enriched in a Ca^{2+}- and temperature-dependent lytic activity, lysing erythrocytes most easily, but also a variety of nucleated cells, in a non-specific manner. The hemolytic activity, which is optimal at neutral pH, is fairly unstable and can be inactivated by exposure of granules of 37 °C and/or to submillimolar amounts of calcium[21]. A T cell hybrid (PC60) that can grow independently of IL-2 becomes cytolytic and acquires cytolytic granules following induction with a combination of IL-1 and IL-2[14,36].

Characterization of perforin

Several laboratories have shown that isolated granules deposited on TC membranes (or lipid vesicles) are capable of causing tubular lesions with an internal diameter of about 16 nm, resembling the previously described polyperforin lesions induced by intact effector cells (for example, see refs[46,75,76], reviewed in Henkart[20]). Granules induce marker release from lipid vesicles[5,9] and rapidly depolarize the membrane potential of nucleated cells[75,76]. The change in membrane resistance of planar lipid bilayers occurs as a progressive incorporation of discrete ion channels, measured as a stepwise increment of current until the membrane breaks down[76]. These results indicate that large, stable, and voltage-resistant channels are formed by granule proteins, attributes that are compatible with an active role for these channels in mediating cytolysis.

Perforin (also called pore-forming protein, PFP, cytolysin, or C9-related protein), the protein responsible for the Ca^{2+}-dependent lytic activity of granules, has been purified from murine, rat and human CTL cell lines, NK cell lines, and stimulated peripheral blood lymphocytes[32,37,48,75,82]. After solubilization of granule proteins with high salt buffers, perforin can be separated from the other proteins by gel filtration chromatography[37], a combination of gel filtration and ion exchange chromatography[48,75,77], or affinity chromatography using an anti-C9 antiserum[80]. Perforin is a hydrophilic polypeptide with an apparent M_r of 65–70 kDa under non-reducing conditions. Reduction results in a shift to 75 kDa presumably due to disruption of internal cysteine bonds.

As demonstrated with whole granules, purified perforin exhibits potent lytic activity against a wide variety of target cells and artificial lipid vesicles in the presence of Ca^{2+}[9,31,75]. Ion conductances are rapidly induced by incorporation of perforin into high resistance membranes[75]. In addition, dyes and macromolecules have been shown to pass the plasma membrane of perforin-treated cells or vesicle lipid bilayers[5,43]. Nucleated cells, which vary considerably in their susceptibility to perforin, generally require 10–100 times more perforin than erythrocytes for lysis to occur. Although the reasons for these sensitivity differences are unknown, they may reflect differences in the capacity to repair membrane lesions or protection by cell-specific inhibitors.

In membranes, perforin is found as a tubular polymer – polyperforin – with an apparent M_r of approximately 10^3 kDa[77]. Electron microscopy of target cell membranes lysed by purified perforin has revealed the presence of typical tubular, ring-like polyperforin structures with inner diameters of 6–16 nm and a height of 16 nm[31,37,48,77]. In the presence of Ca^{2+}, perforin undergoes dramatic ultrastructural changes involving a transition from its monomeric, hydrophilic state into a polymeric, amphiphilic form. The tubules formed by polyperforin contain approximately 12–18 molecules, but complete ring closure is probably not essential for transmembrane channel formation and cell lysis. Pores formed by perforin thus strikingly resemble lesions caused by the complement membrane attack complex (MAC) or the C9 component (for reviews, see Bhakdi et al.[4], Muller-Eberhard et al.[38], Podack and Tschopp[47] and Tschopp et al.[66]).

The primary structures of murine[54], rat[25] and human[31,55] perforin have been independently deduced by cDNA cloning and sequencing. Alignment of the three predicted amino acid sequences reveals considerable identity (69% of the residues are identical between mouse and human perforin). Human perforin is predicted to contain 534 amino acids preceded by a 21-residue leader peptide, as expected for a secreted or granule-stored protein, and three potential glycosylation sites. The difference between the predicted (60 kDa) and the observed M_r (70 kDa) of the protein indicates that carbohydrate moieties account for about 10 kDa of the mature molecule.

Relationship of perforin with the late complement components

The observations of common antigenic epitopes between the lytically active proteins of the humoral defence system (the late complement components comprising the MAC) and those of cytolytic lymphocytes had suggested that they were all derived from common ancestral genes even before pore formation by CTL and the existence of perforin had been demonstrated[61,64,73]. Primary sequence comparison of the central portion of approximately 280 residues of the perforin molecule with protein sequences available in the Swiss Protein Databank identified a homology of about 20% with a region of the

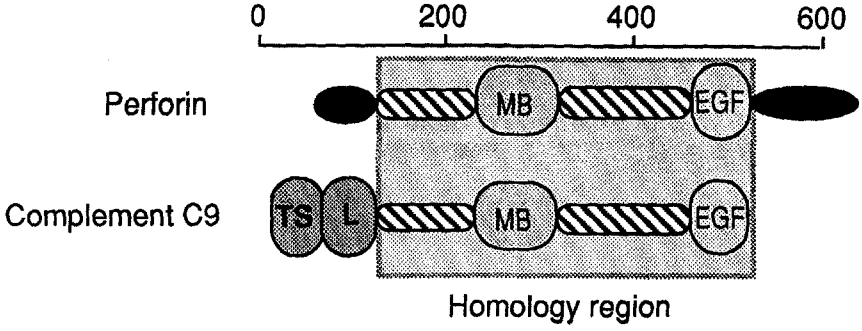

Figure 3. Structural organization of human perforin and the terminal complement component C9. This schematic drawing shows the arrangement of the various structural motifs present in perforin and C9: TS thrombospondin module; L, LDL receptor class A module; EGF, EGF receptor module; MB, candidate lipid binding domain.

complement proteins C6, C7, C8α, C8β, and C9[60]. No detectable homology between perforin and the complement proteins at the N- and C-termini (see Tschopp and Nabholz[65] for a review) or with any other proteins in the databank was found. The central segment is thus predicted to be responsible for the structural and functional similarities between C9 and perforin, in particular for the capacity to polymerize into similar tubules and to insert into membranes. Two previously identified structural elements are found within this region: 1) A putative lipid binding domain (LB in fig. 4, residues 167–211 in perforin, 292–333 in C9) is the most conserved region in the entire sequence and has been proposed to form two amphipathic α-helices that interact with the lipid bilayer (see below). 2) The central segment common to all complement proteins and perforin contains a cysteine-rich domain of the LDL receptor class B (or EGF precursor) type[65]. The function of this module, present in many other molecules, such as coagulation proteins and cell surface receptors, is still not known.

Mechanism of perforin action

Two separable steps have been defined in pore formation by perforin. First, perforin binds to the target membrane and second, it inserts into the lipid bilayer and polymerizes[67, 74]. Perforin binding without membrane insertion occurs at temperatures around 4 °C, provided that the Ca^{2+} concentration is at least 0.1 mM and the pH is above 6.5 (neutral pH is optimal). The insertion step can also be halted after perforin binding in low ionic strength buffers, apparently even at 37 °C[29]. In contrast to simple binding, insertion of perforin into the membrane does not require Ca^{2+} but proceeds optimally at 37 °C in physiological ionic strength buffers, probably because of the energy requirement for the postulated structural rearrangement, or 'unfolding' of perforin that precedes insertion. It has been proposed, as for C9, that upon unfolding of monomeric membrane-bound perforin, initially buried hydrophobic domains are exposed.

Amphipathic elements are likely to be involved in pore formation, since the outside of the pore that interacts with lipids must be hydrophobic, whereas the inside that permits passage of water must be hydrophilic. Secondary structure predictions allow the identification of two amphipathic α-helices separated by a turn in both C9 and perforin[41]. This predicted membrane-spanning region is found within a segment of C9 which has been shown, with a membrane-restricted photoaffinity label, to contain the membrane-interaction site.

Despite their structural similarities, perforin and C9 interact differently with target cell membranes. Whereas C9 insertion into large multilamellar vesicles or erythrocyte membranes is absolutely dependent on a receptor moiety assembled from the complement proteins C5b, C6, C7, and C8 (C5b-8) on the membrane[38], no requirement for a proteinaceous receptor molecule has been reported for perforin. In fact, Tschopp et al.[67] have provided evidence that perforin binds to phosphorylcholine in a Ca^{2+}-dependent manner. They demonstrated that vesicles or lipoproteins containing lipid headgroups such as sphingomyelin and phosphatidylcholine compete with the erythrocyte membrane for the binding of perforin, and hence reduce the hemolytic activity of perforin. Vesicles containing phosphatidylserine or phosphatidylethanolamine did not compete for perforin binding. Moreover, perforin-mediated hemolysis can be inhibited by 1 mM choline phosphate or 3 mg/ml phosphorylcholine-modified bovine serum albumin (corresponding to ~ 1.3 mM phosphorylcholine molecules), whereas no inhibition is caused by ethanolamine phosphate even at 50 mM or by non-modified bovine serum albumin. These results, taken together with direct evidence for binding of perforin to phosphorylcholine in the presence of Ca^{2+} and only at a pH above 6 (conditions which are consistent with those required for perforin's lytic activity)[67], suggest that perforin, upon exocytosis, binds to phosphorylcholine molecules on the target cell membrane.

Role of perforin in the cytotoxic process

Involvement of other granule proteins in cytotoxicity

Controversy still remains as to whether perforin delivery to the target cell by exocytosis is sufficient or even neces-

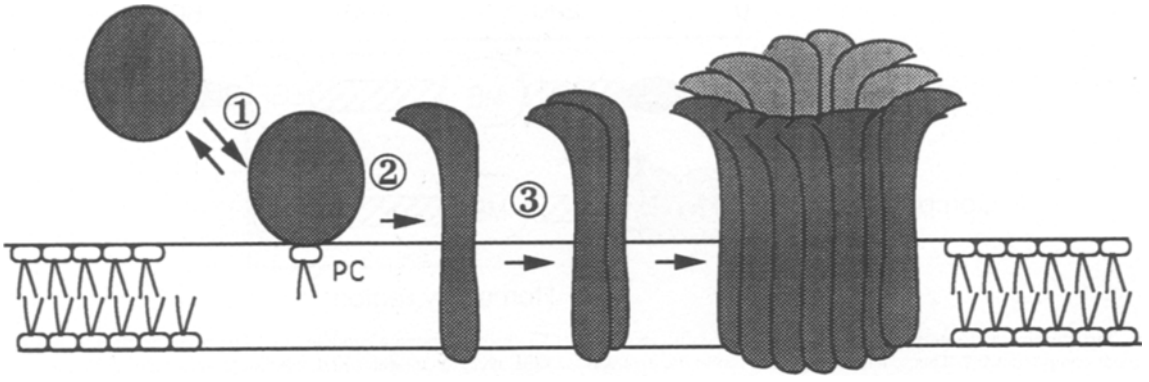

Figure 4. Model of perforin-lipid insertion. Two separable steps have been defined in pore formation by perforin. First, perforin binds to the target membrane and second, it inserts into the lipid bilayer and polymerizes. Perforin binding without membrane insertion occurs at temperatures around 4 °C in the presence of Ca^{2+}. This binding is reversible since withdrawal of Ca^{2+} leads to its detachment. Insertion of perforin into the membrane does not require Ca^{2+} but proceeds optimally at 37 °C. Structural rearrangement, or 'unfolding' of perforin precedes insertion. It has been proposed, as for C9, that upon unfolding of monomeric membrane-bound perforin, initially buried hydrophobic domains are exposed. Finally, up to 16 perforin protomers oligomerize and form a 16-nm wide transmembrane channel.

sary for cell death. Taking into account the data of several groups, the need for additional factors in the perforin-mediated lysis or even the existence of perforin-independent cytolytic mechanisms were postulated. The possibility that several granule components can contribute to the target cell damage was considered since purified perforin, in contrast to whole granule preparations, does not mediate DNA damage. Several groups propose an involvement of granzymes in the induction of apoptosis, since DNA breakdown can be inhibited by serine esterase inhibitors. Following the DNA fragmentation activity during granule fractionation of either mouse CTL or rat natural killer cells, two groups show results which in one case imply a role for granzyme A [18] and in the other for granzyme B [53] in target cell DNA breakdown. In both cases, DNA degradation is only seen if target cells are pretreated either with detergent or perforin. This result suggests that perforin is necessary for the uptake of the DNA degradation mediator into the target cell, although the exact mechanism has not yet been defined.

In analogy to diphtheria toxin, Shi et al. [53] propose that the DNA-damaging molecule could be endocytosed by the target cells, as they try to repair the membrane damage induced by perforin. Another possible route of entry would be by directly passing through the pores created by perforin. Whether granzymes are directly involved in DNA degradation or, what is more probable, function as activators of a cellular DNA degrading machinery, has yet to be investigated.

Recently, Tian et al. [63] isolated a novel protein from cytotoxic granules of human CTLs, named TIA-1, which also induces DNA degradation after detergent permeabilization of the target cell membrane. The protein is structurally related to a family of RNA-binding proteins including poly(A)-binding proteins, ribonucleoproteins and putative transcriptional activators. In cytolytic granules, TIA-1 is mostly present as a 15-kDa isoform, which is thought to be derived from a 40-kDa isoform by proteolytic cleavage. Since granzyme A was shown to specifically cleave nucleolin [40], a protein belonging to the same family, the authors propose a possible role for this serine esterase in processing/activating TIA-1. By what mechanism, however, TIA-1, once activated, induces apoptosis is totally speculative.

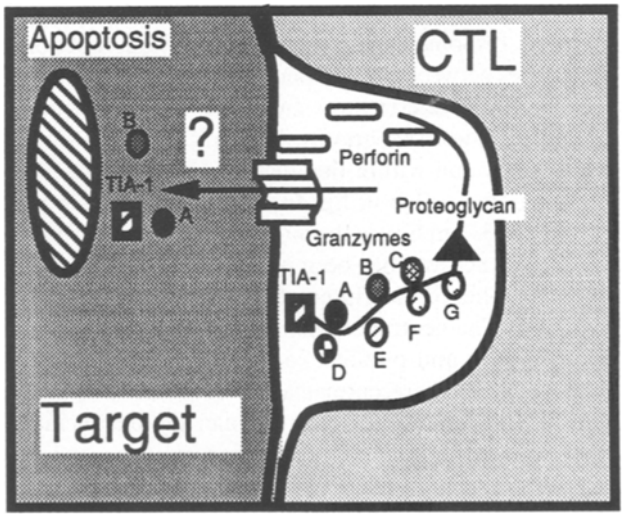

Figure 5. Possible involvement of other granule-associated proteins in cytotoxicity. Three proteins, i.e. granzyme A, granzyme B and TIA-1 have been proposed to act in concert with perforin to induce target cell DNA degradation (apoptotic cell death).

The above data indicate that pore formation by perforin is probably the first step in the cell killing pathway, allowing other granule components to enter the target cell, which then finally activate a genetically determined suicide program. Whether different effector molecules (i.e. granzyme A, B, TIA-1) are used in different species or whether these proteins are involved in the same pathway, but on different levels of action, needs further investigation.

Alternative killing mechanisms

In addition to granule-mediated cytolysis, recent observations suggest the existence of other killing mechanisms. Some CTL lines can kill target cells in the absence of calcium, whereas the exocytosis/perforin-mediated cytolysis is absolutely Ca^{2+}-dependent. Following an intensive search for alternative cytolytic mechanisms, a family of cytolytic proteins, secreted both by CTLs and NK cells, with functional and immunological relationships to tumor necrosis factor, was characterized. In addition to TNF itself, the family includes lymphotoxin [30], leukalexin [33], natural killer cytotoxic factor [68], and possibly other proteins. These factors are able to lyse certain target cells selectively, but more slowly than perforin does. In addition, lymphotoxin and leukalexin can induce DNA fragmentation. Although the importance of the cytotoxic effector molecules described above needs further clarification, it would be feasible that CTL and NK cells use different cell killing mechanisms depending on the target cell type.

Although recent results in several laboratories have strengthened the evidence for the role of perforin and granule exocytosis in T and NK mediated cytotoxicity, all the evidence is still indirect. Acha et al.[1] showed that treatment of CTLs with perforin anti-sense oligonucleotides partially (up to 65%) inhibited their cytolytic capacity. Whether the remaining activity is due to insufficient elimination of perforin mRNA or reflects the presence of alternative killing mechanisms could not be proven. In another approach, Shiver et al.[56] took advantage of the lymphocyte-like, antigen (IgE) receptor-driven exocytic granule pathway of a nonlytic basophilic leukemia cell line. By transfection with a perforin expression vector, this cell line became lytic for IgE-sensitized red blood cells. In contrast to lymphocytes, however, these artificial killer cells were not able to lyse nucleated cells. This inability could be due either to an insufficient release of perforin, since nucleated targets, in general, require higher concentrations of perforin for lysis than anucleated cells, or to the need for additional cytotoxic factors.

In view of the limitations of in vitro experiments, it appeared to us that the most promising way to evaluate the role of perforin would be the generation of a perforin-deficient mouse model, which would provide us on the one hand with perforin-negative lymphocytes for in vitro assays and biochemical analysis, and on the other hand with the whole animal as a powerful tool for more complex in vivo studies.

Perforin gene targetting by homologous recombination

The stable incorporation of cloned copies of genes into eukaryotic cells occurs when the incoming DNA integrates into random sites in the genome or when it undergoes homologous recombination (HR), a process by which it replaces its normal chromosomal homologue. The latter can be exploited to replace endogenous genes with 'HR constructs' containing sequence changes that result in subtle or dramatic changes in the expression or function of the gene [62].

Such direct manipulation of genes in their chromosomal sites provides a valuable means of exploring gene function. The use of this technique in combination with cultured embryonic stem (ES) cells [15] now allows the replacement of normal cellular genes with defined sequence alterations. ES cells are pluripotent cells derived from preimplantation mouse embryos, which can be propagated in culture. HR constructs are introduced into ES cells by electroporation. Because this technique is rather inefficient, a selectable marker must be included in the construct, such as the neomycin phosphotransferase gene, so that cells which do not stably integrate the target DNA can be eliminated. Given the relative infrequency of HR, detection of successfully targetted cells becomes very im-

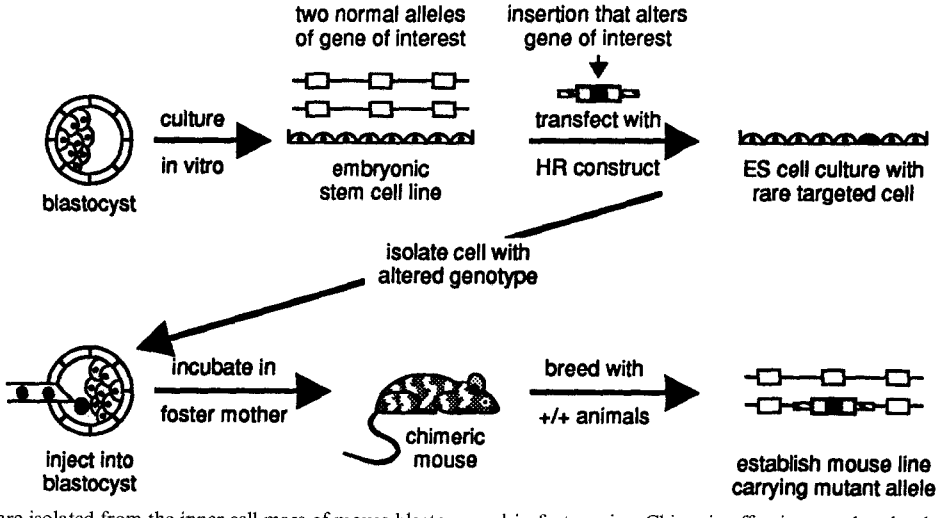

Figure 6. ES cells are isolated from the inner cell mass of mouse blastocysts and transformed in vitro with a targetting construct. ES clones with the mutated genotype are isolated, injected in blastocysts and reimplanted in foster mice. Chimeric offspring are then bred to obtain mice heterozygous or homozygous for the altered allele.

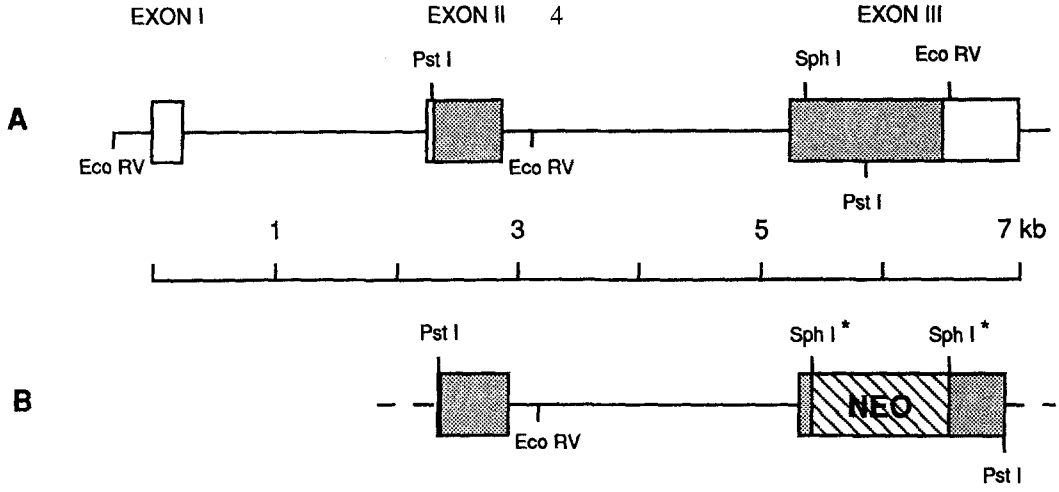

Figure 7. Genomic organization of perforin (A). Targetting construct containing the neomycin resistance gene in the SphI site of the second exon (B). Thin lines represent introns; dashed line vector DNA; pointed boxes, exons; striped box, neomycin-resistant box.

portant. In the absence of a selectable phenotype cells which underwent HR can be isolated by repeatedly subdividing ES cell pools and testing for the presence of cells with the desired change. This can be done very efficiently by PCR, selecting primers such that amplification can occur only when HR has taken place. Primer 1 is chosen from a unique region in the HR construct, and primer 2 from genomic DNA sequences in the gene of interest, but outside the region used for the targetting construct. ES clones containing the disrupted target gene are then reintroduced into mouse blastocysts by microinjection and can contribute to the germ-line of the resultant chimeras.

The advantage of being able to target modifications is that genes are manipulated in their natural chromosomal environment, whereas the use of conventional methods for introducing DNA sequences into the germ-line allows no control over the chromosomal site of integration or the number of copies introduced. At the very least, this complicates the interpretation of gene expression studies, and may result in insertion either into sites that are inappropriate for expression or into essential genes, with deleterious consequences.

Construction of the targetting vector

To construct the targetting vector the 3.4 kb PstI-fragment containing exon II and part of exon III of the perforin gene was subcloned and a pMC1neopA cassette was inserted into the unique SphI-site in exon III. This insertion disrupts the reading frame in a region which shows about 20% of amino acid homology with the proteins of the terminal complement complex.

One identified structural element in this region codes for a putative lipid binding domain and has been proposed to form two amphipathic α-helices that interact with the lipid bilayer. It was thus predicted to be involved in the mechanism of polymerization and membrane insertion of perforin. This vector was used to transform ES cells,

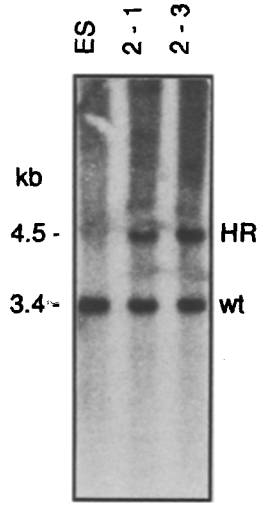

Figure 8. Southern blot analysis of 2 ES clones targetted with the vector shown in figure 7. The upper band (HR) represents the targetted allele, the lower band corresponds to the wild-type allele (wt). In the left panel, a control ES clone is shown.

in which it simulates a correct targetting event. After selection for G418-resistant colonies these cells were used for PCR optimization.

Isolation of successfully targetted ES cell clones

Thirteen independent targetting experiments were carried out using four different ES cell lines. After 10–12 days of selection with G418 several hundred colonies were visible. Single resistant colonies were picked and half of each colony was screened for homologous recombination in a PCR assay as pools of 12 colonies. The other half was expanded in one well of a 48-well plate. As soon as the cells were regrown to confluency, single wells of a positive pool were rescreened by PCR. Successfully targetted clones were then expanded and frozen down. To verify the disruption of one copy of the perforin gene, genomic DNA of the clones was isolated and analyzed by

Southern blotting using an external probe that detects only HR events. Figure 8 shows the results for 2 PCR-positive clones. In addition to the endogenous fragment, a different-sized band of about the same intensity as the wild-type band was found, indicating the targetting of one gene copy.

Generation of chimeric offspring

To generate chimeric mice carrying the perforin deletion, 10–15 cells of targetted clones were injected into C57BL/6 blastocysts. Embryos were reimplanted into the uterus of pseudopregnant mice. Several chimeric mice were born with varying degree of chimerism, but so far, no germline transmission was observed.

Perspectives

The on-going controversy concerning the essential role of perforin in lymphocyte-mediated cytotoxicity continues to be unresolved. There appear to be one or more other mechanisms of lethal hit delivery, possibly acting in concert with perforin or in an independent, alternative manner. The mechanism employed may depend on the nature of the target or effector cell, the presence of accessory cells or the concentration of lymphokines at the site of interaction. The production of mice with a homozygously non-functional perforin gene (by targetted gene disruption) may be the only means of providing a definite answer. If mice with the disrupted gene are still capable of developing lymphocyte-mediated cytotoxic responses, the essential role of perforin will have been ruled out and an experimental system will be available in order to identify the alternative effector molecule(s). On the other hand, if the mice with the disrupted perforin gene no longer exhibit a cytotoxic response, the essential – though not necessarily sufficient – role of perforin will have been proved.

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