Killing of cells by perforin

Resistance to killing is not due to diminished binding of perforin to the cell membrane

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Different cell types vary widely in their susceptibility to killing by the pore-forming cytolytic molecule perforin. In particular, the cells responsible for synthesis of perforin, i.e. cytotoxic T lymphocytes (CTL) and natural killer (NK) cells, are very resistant to cytolysis by this molecule. It has previously been suggested that resistance is due, at least in part, to diminished binding of perforin to these cells. The purpose of the present study was to compare binding of perforin to sensitive and resistant cell types. To this end, perforin was biosynthetically labelled prior to purification. The purified labelled protein was then utilized to obtain a direct measure of the amount of perforin bound to cells during attack. Resistant cells (CTL, neutrophils) bound at least as much perforin as did sensitive cells (K562, HL60 etc.), indicating that resistance to perforin involves mechanisms operating after binding of the lytic molecule.

INTRODUCTION

The granules of cytotoxic T lymphocytes (CTL) and natural killer (NK) cells contain a number of cytotoxic factors which effect target cell killing (Henkart, 1985). One of these factors, termed perforin or cytolytic (Masson & Tschopp, 1985; Podack *et al.*, 1985), is a 70 kDa protein which operates by forming pores in the target cell membrane. In this respect perforin is functionally analogous to the membrane attack complex (MAC) of complement. Formation of the membrane-inserted polyperforin pore involves polymerization of soluble perforin in much the same way that formation of the MAC involves polymerization of C9 (Young *et al.*, 1986a). Sequence analysis of perforin has revealed structural similarities with the component proteins of the MAC, particularly C9 (Lichtenheld *et al.*, 1988; Lowrey *et al.*, 1989). The requirements for perforin pore formation have been examined by several groups using the purified protein. Binding of perforin to cell membranes and its subsequent polymerization both require extracellular Ca^{2+} (Young *et al.*, 1987; Ishiura *et al.*, 1990). Even in the absence of a target membrane, perforin rapidly polymerizes in the presence of Ca^{2+} , thereby irreversibly losing its lytic activity (Young *et al.*, 1986b).

During the delivery of the 'lethal hit', effector cells (CTL or NK) are exposed to concentrations of perforin (and other toxic molecules) which, though sufficient to kill the target, appear to cause minimal disturbance to the effector. This enigma of effector cell survival has initiated a search for the mechanisms by which these cells resist killing. A number of potential mechanisms have already been suggested (Zalman *et al.*, 1987; Young & Liu, 1988), though none has yet gained universal acceptance. Resistance to perforin lysis could occur in several ways: firstly, perforin might fail to bind to the membrane; secondly, evolution of the lytic lesion might be blocked (perhaps by inhibition of perforin polymerization); and thirdly, the lesion might be rapidly and efficiently removed from the cell surface. In cells attacked with the MAC, resistance to lysis involves both the second and third possibilities: inhibitors prevent C9 polymerization and efficient mechanisms of MAC removal allow cells to recover (reviewed in Morgan 1989). To date, no specific inhibitors of perforin have been identified on the membranes of resistant cells, and though we have previously demonstrated that cells may

recover from perforin attack (Jones *et al.*, 1990; Scolding *et al.*, 1990) the mechanisms of recovery remain unclear. A decrease in the amount of perforin binding to resistant cells (CTL) in comparison with susceptible cells has previously been reported and proposed as an important factor in resistance (Jiang *et al.*, 1990a,b). Those studies utilized a competitive lysis system where binding of perforin to the target cell under investigation was assessed by measuring the lysis of erythrocytes admixed with the target. This complicated system was necessitated by the failure of attempts to radiolabel perforin without loss of activity and the lack of high-affinity monospecific antibodies.

In the present study we have utilized biosynthetically labelled perforin to circumvent these problems and to re-assess the binding of perforin to resistant and susceptible cell types. There was no correlation between the amount of perforin bound and killing, with resistant cells (CTL and neutrophils) binding at least as much perforin as sensitive cells (K562, HL60, U937 and Y3 cell lines). The bulk of the cell-bound perforin on both sensitive and resistant targets was present as SDS-resistant polyperforin polymers. Examination of the fate of the bound perforin on resistant cells revealed evidence suggestive of ectocytic elimination from neutrophils but not from CTL.

MATERIALS AND METHODS

General materials

Cysteine-free medium was from Gibco Ltd. (Paisley, Scotland, U.K.). [^{35}S]Cysteine was from Amersham (Aylesbury, Bucks., U.K.). Chromatography materials were from Pharmacia (Milton Keynes, U.K.). All other chemicals were from Sigma (Poole, Dorset, U.K.) or BDH (Poole, Dorset, U.K.) and were of the best grade available.

Hepes buffer contained 0.12 M-NaCl, 4.8 mM-KCl, 1.2 mM- KH_2PO_4 , 1.2 mM- MgSO_4 , 1.3 mM- CaCl_2 , 25 mM-Hepes and 0.1% BSA serum albumin, pH 7.4. PIPEG solution contained 0.1 M-KCl, 3.5 mM-NaCl, 3.5 mM- MgCl_2 , 1 mM-ATP, 1.25 mM-EGTA and 0.01 M-Pipes, pH 6.8. Tris-buffered saline (TBS) contained 0.01 M-Tris and 0.2 M-NaCl, with the pH adjusted to 7.4 with conc. HCl.

Phosphate-buffered saline (PBS) was supplied in tablet form by Oxoid Ltd. (Basingstoke, Hants. U.K.). One tablet was

Abbreviations used: CTL, cytotoxic T lymphocytes; NK cells, natural killer cells; MAC, membrane attack complex; PBS, phosphate-buffered saline; TBS, Tris-buffered saline; DSP, dithiobis(succinimidyl propionate); PMN, polymorphonuclear leucocytes.

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dissolved in 100 ml of distilled water to give 0.137 M-NaCl, 2.7 mM-KCl, 8.1 mM- Na_2HPO_4 and 1.5 mM- KH_2PO_4 , pH 7.3.

Complement fixation diluent was supplied in tablet form by Oxoid Ltd. One tablet was dissolved in 100 ml of distilled water to give 3.1 mM-barbitone, 0.9 mM-sodium barbitone, 0.14 M-NaCl, 1.8 mM- MgCl_2 and 0.3 mM- CaCl_2 , pH 7.2. Gelatin was added to a final concentration of 0.1 %, to give GVB.

Cells

K562 (human erythroleukaemia), U937 (human histiocytic lymphoma), HL60 (human promyelocytic leukaemia) and Y3 (rat myeloma) cell lines were obtained from the European Tissue Culture Collection, Porton Down, Salisbury, U.K., and were maintained in RPMI 1640 (Imperial Labs., Salisbury, U.K.) supplemented with 10 % foetal calf serum (Flow-ICN, High Wycombe, Bucks., U.K.). CTLL2 (CTL), a mouse cytotoxic T cell line, was obtained from the American Tissue Culture Collection, (ATCC), Bethesda, MD, U.S.A. and maintained in RPMI 1640 containing 10 % (v/v) foetal calf serum and 40 % (v/v) rat growth factor as described in the ATCC instructions.

Anti-perforin antibody

Antiserum to mouse perforin was raised in half-lop rabbits by immunization with a synthetic peptide corresponding to amino acids 462–476 of the mouse perforin sequence. The peptide was coupled to keyhole-limpet haemocyanin via dithiobis (succinimidyl propionate) (DSP; Pierce, Cambridge, U.K.) at a peptide/KLH ratio of 5:4 (w/w) prior to immunization of the animals. Sera were tested for reactivity against the coupled and uncoupled peptide and purified perforin by e.l.i.s.a. Antibodies were immunoaffinity-purified on a column of Sepharose 4B (Pharmacia) to which a peptide-BSA DSP conjugate had been coupled. The affinity-purified antibodies were reassessed for binding to peptide and perforin in an e.l.i.s.a.

Biosynthetic labelling of perforin

CTL cell suspension (5 litres) was harvested by centrifugation (900 g, 5 min) when the cell density reached approx. 10^5 /ml (total of 5×10^8 cells). After washing in cysteine-free medium, the cells were resuspended in 50 ml of cysteine-free medium containing 10 % foetal calf serum 40 % rat growth factor, both extensively dialysed against PBS to remove free amino acids. [^{35}S]Cysteine (1 mCi) was added and the cells were placed in a 75 cm³ flask and incubated at 37 °C in an atmosphere of 5 % CO_2 for 5.5 h. These conditions were chosen to maximize labelling of proteins while retaining cell viability at this high cell density. The cells were harvested by centrifugation, washed twice in PBS and once in PIPEG at 4 °C, resuspended in PIPEG (8 ml) and snap-frozen for storage at –70 °C. Granules were isolated and perforin was purified by a modification of the previously published method (Jones *et al.*, 1990), with a second passage over the Mono Q column at a higher pH (8.2) replacing the Superose 6 gel filtration step. In all purification steps, perforin was detected by lysis of guinea pig erythrocytes. The purity of the labelled protein was assessed by SDS/PAGE. Approx. 0.6 μg of each of the three haemolytically active fractions from the Mono Q elution were run, both reduced and non-reduced, on 10 % (w/v) polyacrylamide gels. The gels were soaked in autoradiography enhancer (Enhance; New England Nuclear, Stevenage, U.K.) for 1 h, dried under decreased pressure and placed in a cassette containing film and an intensifier screen. Autoradiographs were kept at –20 °C for up to 14 days prior to development.

Binding of perforin to cells

Cell lines were harvested from tissue culture and washed twice in Hepes buffer at 4 °C. Human neutrophils (PMN) were isolated

from heparinized whole blood by dextran sedimentation, centrifugation (650 g, 25 min) through Ficoll-Hypaque and hypo-osmotic lysis of erythrocytes, as previously described (Morgan & Campbell, 1985). All cell types were adjusted to 6.5×10^6 /ml in ice-cold Hepes buffer. Quadruplicate aliquots of each cell type (200 μl ; 1.3×10^6 cells) were placed in Eppendorf tubes and 0.12 μg of labelled perforin (approx. 320 c.p.m.) was added to each tube. After a 30 min incubation on ice, the tubes were centrifuged at 750 g for 4 min at 4 °C, the supernatants were removed and retained, and the cell pellets were washed twice in 1 ml of ice-cold Hepes buffer. Radioactivity in all supernatants and pellets was measured in a scintillation counter (Tricarb 4000; Packard, Zurich, Switzerland).

Cell viability was measured by Trypan Blue exclusion before and after exposure to perforin. An identical set of tubes was also incubated with labelled perforin, but at 37 °C to enable the cytolytic effect of the dose of labelled perforin on the various cell types to be assessed.

Stability of perforin binding to resistant cells

The fate of bound perforin on those cells which were resistant to killing was examined. CTL or PMN (7×10^6 cells in 0.4 ml of Hepes buffer) were incubated with labelled perforin (1 μg) for 45 min at 37 °C. A 10 μl portion of cell suspension was removed for measurement of cell death by Trypan Blue exclusion and the remainder was centrifuged at 200 g for 4 min to pellet the cells. A 0.3 ml portion of the supernatant was applied to a Sepharose 2B column (24 cm $\times$ 1 cm) equilibrated in TBS. The column was eluted in this buffer and 0.3 ml fractions were collected. In a control experiment labelled perforin (0.75 μg) was diluted to 0.3 ml in TBS, applied directly to the column and eluted as described above. Radioactivity in all column fractions was measured in a scintillation counter.

Polyperforin formation on susceptible and resistant cells

Human erythrocytes (10 %, v/v), PMN (2.2×10^6 /ml), CTL (2.5×10^6 /ml) and K562 cells (2.5×10^6 /ml), all in Hepes buffer, were incubated with labelled perforin (0.2 ml of cell suspension plus 1 μg of perforin) for 30 min at 37 °C in either the presence (5 mM- CaCl_2) or the absence (0.01 M-EDTA) of Ca^{2+} . A 10 μl portion was removed for measurement of cell viability, and the remainder of the cell suspension was centrifuged at 1000 g for 5 min. The supernatant was retained and the cell pellet was washed twice in ice-cold GVB. For the nucleated cell types, the washed pellets were solubilized in 50 μl of SDS sample buffer containing 1 % Triton X-100, boiled for 5 min and centrifuged. (10000 g, 2 min). Erythrocyte pellets were lysed in water after washing, then dissolved in 1 % Nonidet P40 (75 μl) and boiled for 5 min. The solubilized membranes and supernatants were then subjected to SDS/PAGE on 10 % gels under reducing and non-reducing conditions. Gels were dried and autoradiography was performed as described above.

RESULTS

Biosynthetic labelling of mouse perforin

Incubation of CTL in 50 ml of cysteine-free medium containing 1 mCi of [^{35}S]cysteine for 5.5 h at 37 °C resulted in incorporation of radioactivity into cellular proteins without significant loss of cell viability. Granules were extracted from these cells and perforin was purified as described in the Materials and methods section. SDS/PAGE and autoradiography of haemolytically active fractions from the second Mono Q run (pH 8.2) demonstrated two radioactive bands with molecular masses under non-reducing conditions of 58 kDa and 54 kDa (Fig. 1a)

Fig. 1. Characterization of purified biosynthetically labelled ^{35}S -perforin

(a) The peak haemolytically active fractions eluted from the second Mono Q column step (pH 8.2) were run under non-reducing conditions on an SDS/10% polyacrylamide gel (lanes 1–3) and autoradiography was performed. Two bands of approx. 54 and 58 kDa were present in each lane. (b) The same fractions run under reducing conditions are shown (lanes 1–3). Two bands of 60 and 65 kDa were present in each lane. (c) Pooled purified ^{35}S -perforin (4 μg) was run reduced on an SDS/10% polyacrylamide gel and transferred to nitrocellulose, and the immunoblot was developed using a monospecific rabbit antiserum raised to a 15 amino acid peptide deduced from the mouse perforin sequence (lane 2). Prestained molecular mass markers were run in lane 1. The arrows mark the positions of the 5% stacking gels.

and under reducing conditions of 65 kDa and 60 kDa (Fig. 1b). SDS/PAGE and Western blotting followed by immunoprob- ing with the monospecific anti-perforin antibody revealed that both of the bands in the doublet were indeed perforin (Fig. 1c). The higher-molecular-mass band eluted fractionally before the lower-molecular-mass band from the Mono Q column, suggesting that the proteins differed in net charge. Multiple bands have previously been observed in highly purified perforin preparations and attributed to differential glycosylation (Kawasaki *et al.*, 1990). The specific radioactivity obtained was approx. 2700 c.p.m./ μg of protein. Labelled perforin retained full haemolytic activity

when compared with unlabelled perforin prepared by the same method (results not shown).

Binding of labelled perforin to cells

Binding of ^{35}S -labelled perforin to various human, rat and mouse cell types at 4 °C was assessed. Depending on the cell type, between 6.8% and 18.5% of the added perforin became associated with the cells (Table 1). There was no loss in viability of any cell type after incubation with perforin at 4 °C (> 89% viable, as assessed by Trypan Blue exclusion). In a parallel experiment, cells were incubated with the same dose of perforin at 37 °C and viability was measured after 30 min. CTL and PMN were not killed by this dose of perforin, but the viability of all other cells tested was diminished (between 0% and 31% survival; Table 1).

No correlation was evident between perforin binding and susceptibility to lysis.

Perforin polymerization in membranes

SDS/PAGE and autoradiography of solubilized K562 cells, CTL, PMN and erythrocyte membranes was used to examine polymerization of ^{35}S -perforin on these cell types (Fig. 2). In the presence of 5 mM- CaCl_2 , perforin bound to the cells (Figs. 2a–2d, lane 2). Most of the cell-associated perforin was polymerized, with bands remaining in the sample wells and at the stacking gel/lower gel interface. A faint band of approx. 65 kDa, corresponding to monomeric perforin, was also detected on erythrocytes, indicating that polymerization was less complete on these cells. For CTL, PMN and K562 cells not all of the added perforin bound to the cells, the unbound perforin polymerizing in solution, rendering it inactive (Figs. 2a–2c, lane 1). In comparison, erythrocytes appeared to bind all of the added perforin, as no polyperforin band was detected in the cell medium (Fig. 2d, lane 1). When 10 mM-EDTA was present in the perforin-containing medium, there was no perforin binding to any cell type, illustrated by the absence of bands in lane 4 of each gel. All activity remained in the medium, detected as the two monomeric perforin bands of approx. 60 kDa and 65 kDa (lanes 3), confirming that ^{35}S -perforin binding to cells was Ca^{2+} -dependent. Incubation of perforin with PMN, even in the presence of EDTA, led to the degradation of the labelled protein (Fig. 2b, labelled band at gel front in lanes 2 and 3), an observation made only with this cell type.

Samples run under non-reducing conditions produced similar autoradiographs, but with bands at approx. 54 kDa and 58 kDa replacing those at 60 kDa and 65 kDa (results not shown). The presence of high-molecular-mass bands under both non-reducing and reducing conditions indicates that the high-molecular-mass perforin complexes were non-reducible polymers.

Viability, as assessed by measurement of Trypan Blue exclusion (K562 cells, PMN, CTL) or haemoglobin release (erythrocytes) showed that all of the K562 cells, erythrocytes and even the relatively resistant PMN had been lysed at this higher perforin dose, while CTL viability was decreased to 25%.

Non-lethal perforin attack causes vesiculation of PMN

Gel exclusion chromatography on Sepharose 2B (exclusion limit 4×10^7 Da) of supernatant medium from PMN exposed to a sublytic dose of ^{35}S -perforin (< 5% cell death at end point) revealed a peak of ^{35}S activity in the void fractions of the column, together with a second peak eluting in fractions corresponding to a molecular mass of $< 10^7$ Da (identified by Blue Dextran of known molecular mass). Radioactive profiles of medium from CTL suspensions from an identical experiment and of ^{35}S -perforin alone revealed only the second peak, corresponding to polyperforin (Fig. 3). To further exclude the possibility that the

Table 1. Binding of perforin to nucleated cells

Equal numbers of cells (1×10^6 /tube) of six different cell types were incubated with $0.12 \mu\text{g}$ of ^{35}S -perforin (approx. 320 c.p.m.) for 30 min either on ice or at 37°C . The viability of each cell type was measured by Trypan Blue exclusion before and after exposure to perforin at both temperatures. A minimum of 200 cells was counted per sample. The supernatants and washed cell pellets (two 1 ml washes) of those cells incubated with perforin at 4°C were counted for ^{35}S radioactivity post-incubation. The washes contained insignificant amounts of radioactivity. Background radioactivity was subtracted from each reading. The results for ^{35}S -binding are expressed as percentages of total added counts bound to the washed cells and are the means ($\pm$ S.D.) of quadruplicate samples.

Cell type	Viability (%)			Cell-associated ^{35}S at 4°C (%)	$10^{-4} \times$ Perforin molecules bound/cell.
	Initial	After 4°C incubation	After 37°C incubation		
K562	99	97	17	10.4 ± 1.9	5.3 ± 1.0
U937	90	89	31	6.8 ± 1.3	4.0 ± 0.8
HL60	90	89	1	11.1 ± 1.5	6.2 ± 0.8
Y3	98	96	0	16.6 ± 1.6	9.0 ± 0.9
CTLL2	98	98	95	18.5 ± 1.7	11.1 ± 1.0
PMN	98	95	85	8.7 ± 1.0	4.5 ± 0.5

Fig. 2. Perforin polymerization in membranes

(a) CTL, (b) PMN, (c) K562 cells [$(2.2\text{--}2.5) \times 10^6$ /ml] and (d) erythrocytes (10%, v/v) were incubated (0.2 ml of each) at 37°C for 30 min with $1 \mu\text{g}$ of ^{35}S -perforin in the presence (5 mM- CaCl_2) or absence (0.01 M-EDTA) of Ca^{2+} . SDS/PAGE and autoradiography were performed on the cell medium and solubilized cell pellets. Autoradiographs presented are of samples run on gels under reducing conditions. The lanes represent: 1, cell medium + Ca^{2+} ; 2, cell pellet + Ca^{2+} ; 3, cell medium + EDTA; 4, cell pellet + EDTA. The arrows mark the position of the 5% stacking gels.

radioactivity in the first peak from PMN was associated with perforin polymerized in solution rather than membrane-associated polyperforin complexes, a similar experiment was performed in which the PMN were initially incubated with labelled perforin on ice for 30 min, washed and subsequently

incubated at a higher temperature (37°C). A similar column elution profile to that shown for PMN in Fig. 3 was obtained. In addition, when the first peak from PMN was passed through a $0.2 \mu\text{m}$ filter, previously shown not to significantly retain polyperforin, over 70% of the radioactivity was retained on the

Fig. 3. Removal of perforin from PMN following non-lethal attack

Supernatant (0.3 ml) from CTL and PMN that had been incubated with 1 μ g of ^{35} S-perforin (7×10^6 cells in 0.4 ml of Hepes buffer for 45 min at 37 $^{\circ}$ C) were separately applied to a Sepharose 2B column. The eluted fractions (0.3 ml) were counted for radioactivity. As a control, ^{35} S-perforin incubated in buffer (polyperforin) was also applied, eluted and counted. The void volume of the column was 3.5 ml, and fraction 25 corresponds to an apparent molecular mass of 1×10^7 Da, estimated by the elution volume of blue dextran. —, PMN; ---, control; ·····, CTL

filter, suggesting that the bulk of the perforin was associated with particulate matter.

DISCUSSION

In the experiments presented here, we have shown that mouse perforin can be biosynthetically labelled with [^{35} S]cysteine and subsequently purified by standard procedures. This procedure enables the fate of perforin during attack on cells to be examined. Iodination of perforin utilizing derivatized chloramine-T or Iodogen has been attempted, but this yields an inactive product (Jiang *et al.*, 1990b; J. Jones & B. P. Morgan, unpublished work). The biosynthetic labelling of CTL proteins with [^{35} S]methionine has previously been reported (Young *et al.*, 1986c). However, no attempt to isolate active perforin from the cells was made. Due to the relatively short incubation time of the CTL in radioisotope-containing medium, only a small percentage of the purified perforin will be labelled with [^{35} S]cysteine. The assumption has been made that labelled and unlabelled perforin are identical in their ability to bind to cells. This assumption is justified because biosynthetic labelling of proteins with ^{35} S-labelled amino acids is a well-established technique which, unlike iodination, causes little or no damage to the protein and also because the purified labelled perforin retained haemolytic and cytolytic activity.

^{35} S-perforin was used to study perforin binding to CTL, K562, U937, HL60 and Y3 cells and human PMN, and showed that there was no correlation between the susceptibility of cells to perforin-mediated lysis and their ability to bind perforin at 4 $^{\circ}$ C. SDS/PAGE and autoradiography of cell membranes and medium after incubation of cells with ^{35} S-perforin at 37 $^{\circ}$ C in the presence and absence of Ca^{2+} showed that the association of ^{35} S radioactivity with cell membranes was Ca^{2+} -dependent and not due in any part to non-specific absorption of perforin. We were initially surprised by the amount of perforin binding to CTL at 4 $^{\circ}$ C. It has been reported by Jiang *et al.* (1990a), using a competition assay between indicator sheep erythrocytes and the test cell, that CTL bound much less perforin (about 10-fold less) than did a number of tumour cell lines, including K562 cells. The results presented here demonstrate that CTL did not bind less perforin than other cell types at 4 $^{\circ}$ C on a cell-for-cell basis. On the contrary, CTL bound more perforin than five other cell types

examined. CTL are relatively small cells, particularly in comparison with K562 cells, and if cell surface area had also been taken into consideration the increased binding of perforin to CTL would have been even more remarkable. Our results clearly show that cell susceptibility to perforin-mediated lysis and perforin binding are not positively correlated.

The discrepancies between our results and those of Jiang *et al.* (1990a) were investigated by performing the published competition assay on the CTL and K562 cell lines used in the ^{35} S-perforin binding assay. In our hands it was not possible to compare the binding of perforin between CTL and K562 cells using the described competition assay, as the CTL caused 100% lysis of the indicator sheep erythrocytes even in the absence of added perforin. The phenomenon was even observed after extensive washing of the CTL cells and was presumably due to the release of sufficient CTL granule contents into the supernatant during the assay to cause lysis in the absence of exogenous perforin. In contrast, with K562 cells a good correlation was obtained between increasing cell number and decrease in percentage erythrocyte lysis, as reported by Jiang *et al.* (1990b) (results not shown).

On all cell types examined, the bulk of the bound perforin was present in the form of high-molecular-mass SDS- and reduction-resistant complexes. On erythrocytes, a proportion of the bound perforin remained unpolymersized (Fig. 2d). Perforin incubated with PMN, even in the presence of EDTA, was partially degraded (low-molecular-mass bands at gel front in Fig. 2b), suggesting that these cells released proteolytic enzymes capable of digesting the molecule. The demonstration of ^{35} S-perforin in association with PMN membrane fragments following non-lethal attack suggested the possibility that these cells, which are markedly resistant to perforin attack, may also protect themselves by removing the protein by ectocytic processes (Stein & Luzio, 1991). PMN have been shown previously to recover from non-lethal complement attack via vesiculation of membrane fragments bearing many MACs (Campbell & Morgan, 1985; Morgan *et al.*, 1987). Several other cell types have subsequently been shown to remove MACs by vesiculation (Morgan, 1989). More recently, we have shown that rat oligodendrocytes vesiculate in response to non-lethal perforin attack (Scolding *et al.*, 1990). Thus vesiculation appears to be a common mechanism of resistance to these pore-forming toxins, at least in some cell types.

The marked resistance of CTLs to perforin lysis suggests the presence of a specific membrane inhibitor. Several groups have attempted to identify this inhibitor (Zalman *et al.*, 1987; Jiang *et al.*, 1988; Ojcius & Young, 1990), but the molecule(s) responsible remain unidentified. Although the present study does not identify the inhibitor, it does provide some clues about how it may function. Using very large doses of perforin it was possible to bring about significant lysis even of CTL. At these lytic doses of perforin, when up to 75% of CTL were lysed, most of the cell-associated perforin was present in the form of high-molecular-mass complexes, indicating that perforin readily polymerizes in CTL membranes. Little or no non-polymerized perforin was present on these cells, implying that the putative inhibitor not only allows perforin binding but also permits its polymerization. Regulation of lysis must therefore involve alteration in the structure of the bound polyperforin, such that no functional pore is formed. With higher doses this putative inhibitor may be 'swamped out', allowing sufficient functional polyperforin lesions to form to precipitate cell lysis. The data therefore suggest that the perforin inhibitor operates at a later stage in lesion assembly than does the MAC inhibitory protein, CD59 antigen, which works by limiting C9 input and preventing C9 polymerization (Meri *et al.*, 1990; Rollins & Sims, 1990).

Our results indicate that different cell types resist perforin lysis by different means. While PMN appear to resist lysis, at least in part, by vesiculation (and perhaps by degradation of perforin), no such mechanism was apparent in CTL. We suggest that there is an inhibitor present on the CTL membrane that, while permitting perforin binding and polymerization, prevents functional pore expression.

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