Biochem. J. (1990) 267, 303–307 (Printed in Great Britain)

Reversible cell damage by T-cell perforins

Calcium influx and propidium iodide uptake into K562 cells in the absence of lysis

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The non-lethal effects of the lymphocyte-derived pore-forming toxin perforin on the human erythroleukaemia cell line K562 were investigated. By using the fluorescent Ca^{2+} indicator fura-2, perforin was shown to cause intracellular Ca^{2+} concentration to rise transiently into the micromolar range in the absence of cell death. By fluorescence-activated cell sorting it was demonstrated that K562 cells took up the membrane-impermeant nuclear stain propidium iodide (PI) when exposed to non-lethal doses of perforin. The permeability to PI was short-lived, confirming the transience of the perforin pore. Analogies with non-lethal effects and recovery processes occurring in nucleated cells exposed to the membrane-attack complex of complement are drawn.

INTRODUCTION

The granules of cytotoxic T-lymphocytes (CTLs) contain several active molecules involved in cytolysis of target cells (Podack & Konisberg, 1984). Cells lysed *in vitro* by these granules exhibit ring-like lesions with an electron-microscopic appearance similar to the lesions formed by the membrane-attack complex (MAC) of complement (Podack & Dennert, 1983). Purification of the granule constituents has revealed that the ring lesions are caused by a single protein, perforin. Perforin, which is present in the granules as a monomer of about 70 kDa, self-polymerizes on target-cell membranes in the presence of Ca^{2+} ions to form the ring lesions and effect cell lysis. Perforin-mediated cell lysis is highly analogous to lysis by the MAC, formation of a functional pore through the membrane allowing water and ions to enter the cell, causing the cell to swell and eventually to burst (Podack *et al.*, 1985; Young *et al.*, 1986; Zalman *et al.*, 1986).

We and others have demonstrated that nucleated cells, unlike aged erythrocytes, are able to resist lysis by the MAC and may be stimulated to release active metabolites (Hallett *et al.*, 1981; Campbell & Morgan, 1985; Carney *et al.*, 1985; Morgan *et al.*, 1987; Morgan, 1989). Resistance mechanisms and non-lethal effects are mediated, at least in part, by a transient increase in intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) (Morgan & Campbell, 1985; Carney *et al.*, 1986). The aim of the present study was to establish whether perforin, like the MAC, caused non-lethal changes in nucleated cells. The target cell chosen, the human erythroleukaemia cell line K562, is known to be susceptible to lysis by perforin. We examined changes in $[Ca^{2+}]_i$ and permeability to propidium iodide (PI) in K562 cells exposed to non-lethal amounts of perforin. As previously demonstrated after MAC attack, perforin caused a transient increase in $[Ca^{2+}]_i$ and permitted entry of the membrane-impermeant dye PI into the cell in the absence of cell lysis. The time during which the cells were permeable to PI was short, indicating that functional 'pores' on the cell surface were transient.

MATERIALS AND METHODS

Materials

Fura-2 acetoxymethyl ester (fura-2/AM) was purchased from Molecular Probes (Junction City, OR, U.S.A.). PI was from Sigma Chemical Co. (Poole, Dorset, U.K.). All other chemicals were from BDH Chemicals (Poole, Dorset, U.K.) or from Sigma, and were of the best grade available. Hepes buffer contained 0.14 M-NaCl, 0.025 M-Hepes, 0.005 M-KCl, 0.0013 M-$CaCl_2$, 0.0012 M-KH_2PO_4, 0.0012 M-$MgSO_4$ and 0.1% bovine serum albumin, adjusted to pH 7.4 with NaOH.

The K562 cell line was obtained from the European Animal Cell Collection (Porton Down, Wilts., U.K.) and was maintained in RPMI medium containing 10% (v/v) fetal-calf serum.

Purification of perforin

Perforin was purified from the murine cytotoxic T-lymphocyte cell line CTLL-2 B1/6 (American Type Tissue Culture Collection, Bethesda, MD, U.S.A.) by minor modifications of published methods (Podack *et al.*, 1985). Briefly, 4×10^8 cells were disrupted by nitrogen cavitation. The released granules were fractionated on a Percoll gradient, disrupted by suspension in 1 M-phosphate buffer, pH 7.4, and the released perforin was purified to homogeneity by ion-exchange chromatography (Mono Q) and gel filtration (Superose 6) on a fast protein liquid chromatography (f.p.l.c.) system (Pharmacia). Activity was assayed by measuring haemolysis of chicken erythrocytes.

Titration of non-lethal perforin dose on K562 cells

In order to identify the maximum dose of perforin causing no lysis of K562 cells (non-lethal dose), cells (100 μl of cells at 5×10^6/ml in Hepes buffer) were incubated with various doses of perforin at 37 °C for 60 minutes. Cell lysis was estimated by Trypan Blue exclusion (1000 Da) or by measuring release of lactate dehydrogenase (LDH).

The susceptibility of K562 cells to perforin lysis varied considerably from day to day, perhaps reflecting cell-cycle-dependent factors. The non-lethal dose was therefore determined before each experiment.

Measurement of $[Ca^{2+}]_i$

In order to measure $[Ca^{2+}]_i$, K562 cells were loaded with fura-2 by incubation of cell suspensions [$(1–3) \times 10^7$ cells/ml in Hepes buffer] for 30 min at room temperature with fura-2/AM (final concn. 1 μM). The fura-2/AM was rapidly de-esterified intracellularly, trapping the membrane-impermeant fura-2 acid,

Abbreviations used: $[Ca^{2+}]_i$, intracellular free calcium concentration; CTL, cytotoxic T-lymphocyte; FACS, fluorescence-activated cell sorting; LDH, lactate dehydrogenase; MAC, membrane-attack complex of complement; PI, propidium iodide.

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a fluorescent tetracarboxylic acid Ca^{2+} chelator, in a uniform distribution within the cell. Fura-2 fluorescence emission at 505 nm was measured after excitation at 340 and 380 nm in the Spex II Fluorolog fluorimeter (Glen Spectra, Stanmore, Middx., U.K.), and $[Ca^{2+}]_i$ was calculated from the ratio as previously described (Al-Mohanna & Hallett, 1988). Incubation of loaded cells with digitonin and EGTA provided maximum and minimum calibration signals respectively. Calibration was accurate for Ca^{2+} concentrations up to 5 μM.

Cells loaded with fura-2 were incubated at 37 °C with various non-lethal or low-lethal doses of perforin, and the $[Ca^{2+}]_i$ was continuously monitored in the fluorimeter over a 4 min time course. At the end of the time course, cell death was estimated by measuring LDH release and Trypan Blue exclusion.

Measurement of PI permeabilization by fluorescence-activated cell sorting (FACS)

PI is a membrane-impermeant nuclear dye (668 Da) commonly used as an indicator of cell death. K562 cells (10^6 cells in 1 ml of Hepes buffer) were incubated with PI (final concn. 10 μg/ml) and a non-lethal concentration of perforin (determined beforehand by titration) at 4 °C. The cells were then immediately analysed on a FACS 440 fluorescence-activated cell sorter (Beckton–Dickinson) over a time course of 10 min, by exciting with the 488 nm line of an argon laser and collecting fluorescence emission with a 620 nm long-pass filter. Data on PI fluorescence, forward light scatter (a measure of cell size) and wide-angle light scatter (a measure of granularity) were collected on a microcomputer (Hewlett Packard).

In separate experiments cells were preincubated with a non-lethal dose of perforin at 37 °C for various times before addition of PI and analysed on the FACS 440 5 min after PI addition.

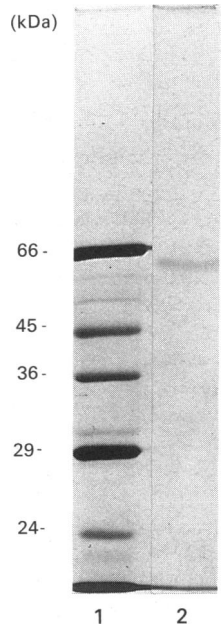

Fig. 1. Assessment of perforin purity

Purified murine perforin from CTLL-2 B1/6 cells was run on a SDS/10%-polyacrylamide gel under reducing conditions. Protein was detected by silver staining. Lane 1, molecular-mass markers; lane 2, purified perforin (2 μg).

RESULTS

Assessment of perforin purity

SDS/PAGE of purified perforin under reducing conditions revealed a single band on silver staining of 65 kDa (Fig. 1).

Non-lethal perforin attack causes a transient increase in $[Ca^{2+}]_i$ in K562 cells

In fura-loaded K562 cells attacked with non-lethal doses of perforin, $[Ca^{2+}]_i$ rose after a short lag from a resting level of 80–110 nM to a peak within 2 min. The measured $[Ca^{2+}]_i$ then fell to a plateau close to the resting level within 5 min of perforin addition (Fig. 2). The lag phase, peak $[Ca^{2+}]_i$ and apparent $[Ca^{2+}]_i$ at the plateau were all dose-dependent, the lag decreasing with increased perforin dose and the peak and plateau $[Ca^{2+}]_i$ rising. By washing the cells after attack it was shown that the increased plateau $[Ca^{2+}]_i$ evident at higher perforin doses was partly the result of leakage of fura-2 from the cells during perforin attack. Washing away extracellular fura-2 decreased the plateau level by about 30%.

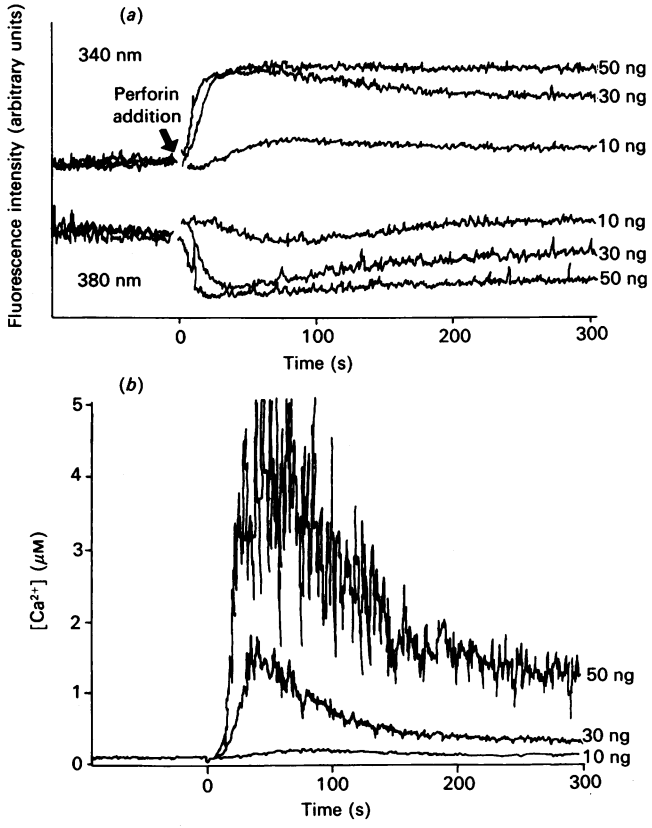

Fig. 2. $[Ca^{2+}]_i$ changes in K562 cells attacked with non-lethal perforin doses

K562 cells (10^6 cells in 1.5 ml of Hepes buffer) were loaded with fura-2, warmed to 37 °C and placed in the Spex instrument (zero time). Perforin was added (zero time) and $[Ca^{2+}]_i$ measured over the following 300 s. Viability by Trypan Blue exclusion was measured at 300 s and was greater than 95% for all perforin doses shown. Data are representative of three separate experiments. (*a*) The upper three traces show fluorescence emission at 505 nm after excitation at 340 nm (Ca^{2+}-bound) and the lower three traces emission after excitation at 380 nm (unbound fura-2). Doses of perforin used were 10, 30 or 50 ng. (*b*) The calculated $[Ca^{2+}]_i$ derived from the above ratios as previously described (Al-Mohanna & Hallett, 1988).

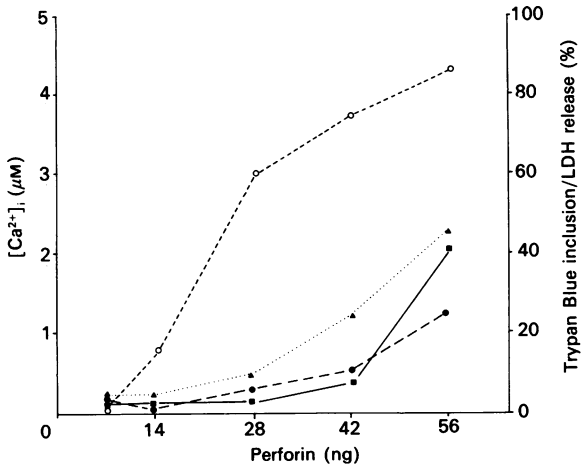

Fig. 3. Response of K562 cells to various perforin doses: $[Ca^{2+}]_i$ changes, LDH release and Trypan Blue uptake

K562 cells loaded with fura-2 (10^6 cells in 1.5 ml of Hepes buffer) were warmed to 37 °C and placed in the fluorimeter. Perforin was added (zero time) and $[Ca^{2+}]_i$ measured over the following 4 min. Samples were taken at 240 s for immediate measurements of LDH and Trypan Blue exclusion. Data presented are representative of three separate experiments. Peak $[Ca^{2+}]_i$ (○), apparent plateau $[Ca^{2+}]_i$ at 240 s (▲), LDH release (%; ■) and Trypan Blue uptake (%; ●) are shown for each perforin dose.

Relationship of $[Ca^{2+}]_i$ changes with cell death

The experiments described above were performed with doses of perforin which caused no cell lysis. In order to determine the relationship between $[Ca^{2+}]_i$ and cell death, Ca^{2+} was also measured in cells subjected to perforin doses which caused cell lysis. Five perforin doses were chosen for these experiments, three of which (7, 14 and 28 ng) caused no cell lysis and two of which (42 and 56 ng) caused limited lysis. As shown in Fig. 3, transient elevations of $[Ca^{2+}]_i$ were seen at all non-lethal doses, reaching a peak of 3 μM with the maximum non-lethal dose. Addition of 42 ng of perforin caused a peak $[Ca^{2+}]_i$ of 3.8 μM, yet viability, assessed by Trypan Blue exclusion or LDH release, was still 90%. The highest dose (56 ng) used caused 30–40% cell death, $[Ca^{2+}]_i$ rising to over 5 μM (the upper limit of accurate measurement).

Non-lethal perforin attack allows entry of PI into K562 cells

FACS analysis of K562 cells incubated with non-lethal amounts of perforin in the presence of the membrane-impermeant nucleic-acid-intercalating dye PI demonstrated that more than 90% of the cells became permeable to the dye (Fig. 4). Increased fluorescence was detectable within 1 min of adding perforin. Because FACS technology incorporates a temporal delay of 30–60 s between sample application and laser interception of the cells, perforin was added to cells at 4 °C to slow down the initial reaction and allow observation of the early events. The cells were warmed to 37 °C once on the machine. The 1 min lag before detectable PI uptake therefore probably represents an overestimate of the time required for permeabilization at 37 °C. PI fluorescence reached a maximum by 6 min and remained at this level for the duration of the experiment (10 min). The uptake of PI has frequently been used as an indicator of cell death, but here the PI-positive cells remained > 90% viable for at least 24 h after uptake, as assessed by measuring Trypan Blue exclusion on PI-positive cells returned to culture after being sorted on the FACS.

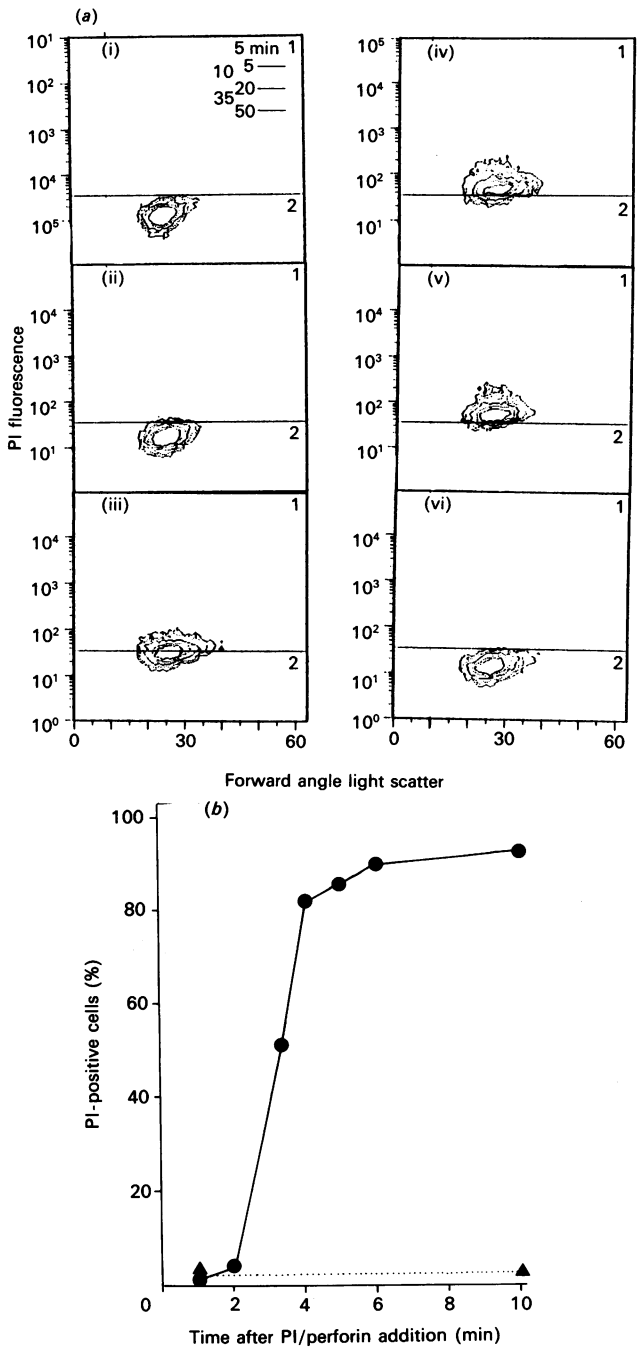

Fig. 4. PI uptake by K562 cells attacked with non-lethal perforin doses

K562 cells (10^6 cells in 1 ml of Hepes buffer) were mixed with PI (final concn. 10 μg/ml) and a non-lethal dose of perforin at 4 °C, then immediately analysed on a FACS 440 at 37 °C over a time course of 10 min. The cells were excited with the 488 nm line of an argon laser, and emission was collected with a 620 nm long-pass filter. Data on PI fluorescence and forward light scatter were collected on a microcomputer. Data are representative of two separate experiments. (*a*) Contour data of PI fluorescence and forward light scatter at various times after perforin addition. Data were collected between 1 and 10 min after addition of perforin and PI and are shown as contour diagrams with PI fluorescence on the ordinate (arbitrary units, log scale) and forward light scatter on the abscissa (arbitrary units). The contour levels are shown in panel (i). Control cells had no perforin added. (i) 1 min; (ii) 2 min; (iii) 3 min; (iv) 5 min; (v) 10 min; (vi) control cells at 10 min. (*b*) The number of PI-positive cells was calculated from the contour data [see dividing line in (iv) above] and the percentage of PI-positive cells (ordinate) was plotted against time after perforin addition (abscissa): ●, perforin added ▲, no perforin.

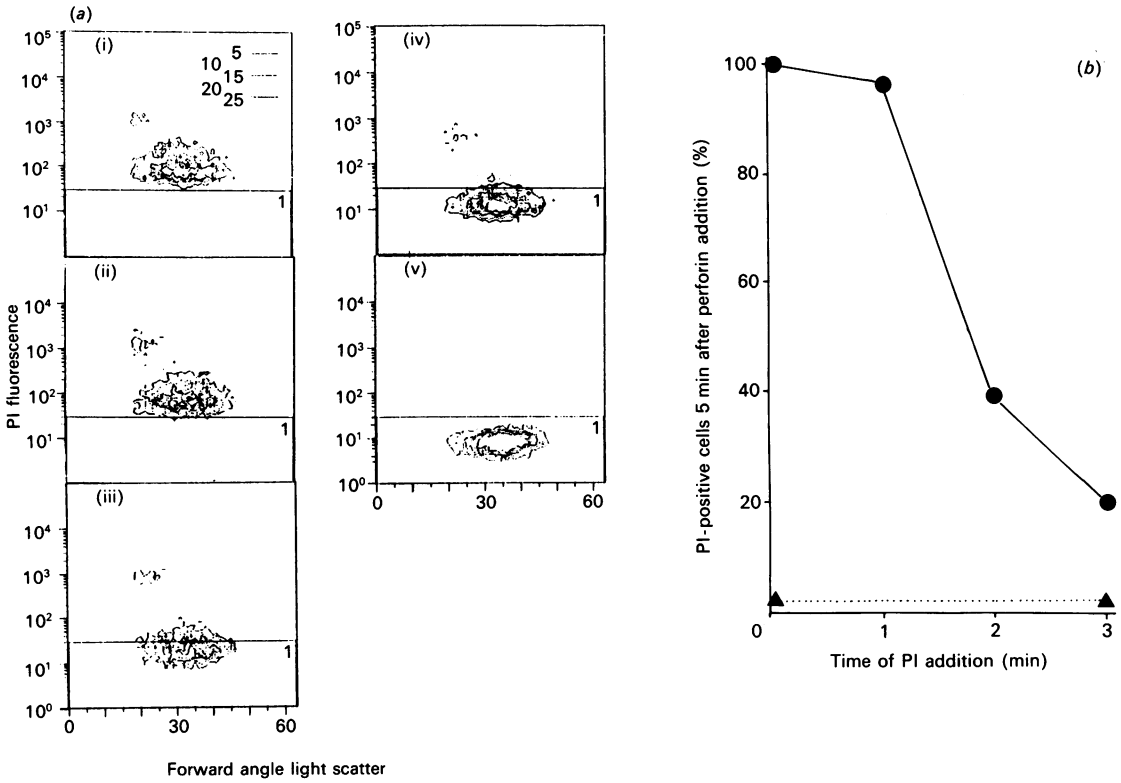

Fig. 5. Transience of PI permeability in K562 cells attacked with non-lethal perforin doses

K562 cells (10^6 cells in 1 ml of Hepes buffer) were incubated with a non-lethal dose of perforin at 37 °C. PI (final concn. 10 μg/ml) was added either at the time of perforin addition (zero time) or at 1, 2 or 3 min after. The cells were analysed on the FACS 440 at 37 °C 5 min after perforin addition. Control cells had PI added at zero time, but no perforin. (*a*) Contour diagrams of PI fluorescence (ordinate; arbitrary units, log scale) and forward angle light scatter (abscissa, arbitrary units) of K562 cells 5 min after perforin addition with PI added at (i) 0 min, (ii) 1 min, (iii) 2 min or (iv) 3 min; (v) control cells. The contour levels are shown in panel (i). A small number of highly fluorescent free nuclei (top left of plots) was present at all times. The horizontal line represents the division between fluorescent and non-fluorescent cells, based on fluorescence in control cells. (*b*) Effect of delayed PI addition. The number of PI-positive cells was calculated from the data above. The percentage of cells positive at 5 min after perforin addition (ordinate) was plotted against time of PI addition (abscissa): ●, perforin added; ▲, no perforin. Data are representative of two similar experiments.

Perforin-induced PI permeability is transient

In order to estimate the lifetime of the functional channel formed by non-lethal amounts of perforin in K562 cells, cells were incubated with perforin at 37 °C and PI was added at various times. As shown in Fig. 5, the amount of PI taken up by the cells started to decrease between 1 and 2 min after perforin addition, and when PI was added 3 min after initiation of perforin attack uptake was minimal, demonstrating that the channel is transient, with a half-life of about 90 s.

DISCUSSION

We here demonstrate that perforin channels in nucleated cell membranes, like those caused by the MAC, are transient, channel elimination or inhibition allowing the cell to escape limited attack. Nucleated cells escape MAC lysis by Ca^{2+}-dependent recovery processes which physically remove the damaging complex from the membrane (Campbell & Morgan, 1985; Carney *et al.*, 1985; Morgan *et al.*, 1987). Preliminary evidence suggests that similar mechanisms operate to eliminate perforin channels.

Although lethal attack of CTLs, natural killer cells, their isolated granules and purified perforin on target cells has been well documented, far fewer experiments have been performed to examine non-lethal attack. Others have reported leakage of ions and transient depolarization after perforin (cytolysin) attack on Lettre cells, implying reversible injury (Bashford *et al.*, 1988). Rapid changes in $[Ca^{2+}]_i$ have been demonstrated in nucleated cells incubated with CTLs and isolated granules (Poenie *et al.*, 1987), and a transient increase in $[Ca^{2+}]_i$ in target cells exposed to sub-lytic doses of granules has been described (Allbritton *et al.*, 1988). However, neither of these studies utilized purified perforin, thereby making it impossible to eliminate contributions of other granule constituents, and results in the crucial first 30 s of non-lethal attack were not obtained.

Our results, obtained with highly purified perforin, shed new light on perforin action on nucleated cells and demonstrate further analogies with the MAC (Patel & Campbell, 1987). Non-lethal attack resulted in the formation of transient channels on the cell surface of sufficient size to allow passage of Ca^{2+} and PI (668 Da) but not of LDH (134000 Da). A rapid increase in $[Ca^{2+}]_i$ occurred, probably as a result of influx via these channels. The possibility of a contribution from intracellular stores could not be eliminated, because removal of extracellular Ca^{2+} abolished perforin activity.

The MAC has also been shown to cause increased $[Ca^{2+}]_i$ as one of the earliest detectable intracellular events, most of the Ca^{2+} being derived from outside the cell, although in nucleated cells some release from intracellular stores also occurred (Campbell *et al.*, 1981; Morgan & Campbell, 1985; Carney *et al.*, 1986). This MAC-induced increase in $[Ca^{2+}]_i$ has been implicated in the stimulation of release of pro-inflammatory factors, in-

cluding prostaglandins and reactive oxygen metabolites from a variety of nucleated cells *in vitro* (reviewed by Morgan, 1989). Pro-inflammatory non-lethal effects may thus also be stimulated in nucleated cells by T-cell/perforin attack. Perforin may thus be added to the list of agents which are implicated in cell stimulation and the perpetuation of the inflammatory response *in vivo*.

We are grateful to the Wellcome Trust and the Arthritis and Rheumatism Council for financial support. B.P.M. is a Wellcome Senior Clinical Fellow.

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Received 2 October 1989/1 December 1989; accepted 7 December 1989