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The membrane attack complex of complement induces permeability changes via thresholds in individual cells

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SUMMARY

Flow cytometry was used to quantify the fluorescence of propidium iodide in rat polymorphonuclear leucocytes (PMN) attacked by the membrane attack complex (MAC) in order to establish the existence of permeability and lytic thresholds in individual cells, a 'threshold' being defined as a cellular event involving the rapid transition of cells from one state to another under physiological conditions. Activation of the complement pathway resulted in PMN being attacked by MAC within 5 min. Approximately 30–40% of the cell population subsequently became permeable to small molecules and macromolecules. Individual PMN passed through 'thresholds' of cell permeability and cell lysis, or recovered from complement attack at different times. In the flow cytometer, three distinct populations of PMN were identified: (i) cells that had recovered before the permeability 'threshold', (ii) cells that had recovered after the permeability 'threshold' but before the lytic 'threshold', and (iii) cells that failed to recover from complement attack. Individual PMN attacked by MAC passed through permeability and lytic thresholds at different times after an initial lag of $7{\cdot}5 \pm 2{\cdot}5$ min and $11{\cdot}5 \pm 1{\cdot}0$ min, respectively. Adenosine, an activator of adenylate cyclase, inhibited removal of MAC from the cell surface. Consequently, more cells passed through the permeability and lytic 'thresholds', resulting in an increased percentage of lysed cells.

INTRODUCTION

Activation of the classical or alternative pathway of complement leads to the assembly of the membrane attack complex (MAC) on the cell surface (Biesecker, 1983; Muller-Eberhard, 1984; Mayer *et al.*, 1981). This complex consists of C5b, C6, C7, C8 and up to 18 C9 molecules (Tschopp, Engel & Podack, 1984). The final stage in the formation of the complex involves insertion of C9, initially a soluble globular glycoprotein of molecular weight 71,000, into the membrane, followed by polymerization of C9 molecules (Podack, Tschopp & Muller-Eberhard, 1982; Stanley *et al.*, 1986). We have shown that within 5 seconds of C9 binding to C5b-8 in the membrane, there is a rapid increase in intracellular free Ca^{2+} (Campbell *et al.*, 1981). This rise in cytoplasmic free Ca^{2+} can stimulate cell responses (Campbell & Luzio, 1981; Hallett, Luzio & Campbell, 1981) and activate processes that can lead to removal of the potentially lethal MACs from the cell surface via vesiculation (Campbell & Morgan, 1985; Morgan & Campbell, 1985; Morgan, Dankert & Esser, 1987). The increase in free Ca^{2+} is followed by an increase in membrane permeability to small molecules and eventually cell lysis, unless the cell has protected itself in time by removing the MACs.

The precise molecular basis of the increases in membrane permeability to cations, small molecules and macromolecules induced by C9, and in particular the role of polymeric C9, has yet to be fully established. A further problem is that most quantitative studies have involved measuring the mean value of a particular parameter from a large cell population. We have shown using flow cytometry that, in avian erythrocytes, the time–course of macromolecule release is the result of individual cells reaching a critical threshold point at different times following complement attack, after which they lyse within a second (Edwards *et al.*, 1983). Two questions therefore arise. Firstly, do increases in the permeability to ions and small molecules of the nucleated cell membrane also involve a threshold in individual cells? Secondly, does inhibition of the protection mechanisms by substances such as adenosine increase the number of cells crossing the reversible permeability threshold as well as the irreversible threshold to cell death (Roberts, Morgan & Campbell, 1985)?

The aim of the work reported here was to study changes in the permeability of individual cells using the nuclear stain propidium iodide. This is normally impermeant to cells and becomes fluorescent only when it intercalates with nucleic acids. It can therefore be used as a probe to determine an increase in the permeability of the cell membrane. Increases in the permeability of individual cells induced by MAC were analysed by flow

Abbreviations: FITC, fluorescein isothiocyanate; MAC(s), membrane attack complex(es); PMN(s), polymorphonuclear leucocyte(s).

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cytometry, and related to the molecular events following C9 binding.

MATERIAL AND METHODS

Chemicals

Propidium iodide, fluorescein isothiocyanate (FITC) and 2-chloroadenosine were purchased from Sigma Chemicals, Poole, Dorset. Ficoll–Hypaque and Sephadex G25 (medium) were obtained from Pharmacia Fine Chemicals, Uppsala, Sweden. Sodium caseinate (Nutrose) was purchased from Difco Laboratories, Detroit, MI. All other chemicals were of 'Analar' grade and were purchased from BDH Chemicals, Poole, Dorset.

Antisera

Rabbit anti-rat liver 5′-nucleotidase serum was prepared according to the method of Stanley, Edwards & Luzio (1980). The serum was placed at 56° for 30 min to inactivate complement and stored in portions at −20°.

Complement

Human blood from normal volunteers was clotted, and the serum used as a source of complement was stored in portions at −70°.

Preparation of labelled antibodies

A mouse monoclonal IgG antibody (coded MC9-36) to human C9 was produced as previously described (Morgan *et al.*, 1983). This antibody has been shown to recognize C9 after its insertion into the MAC on cells (Morgan *et al.*, 1984a, b). FITC-labelled antibody was used to visualize MACs on PMN by fluorescence microscopy.

Preparation of rat polymorphonuclear leucocytes

Rat polymorphonuclear leucocytes (PMN) were prepared from the peritoneal exudate of Wistar rats (200–300 g) 12–14 hr after intraperitoneal injection of sterile sodium caseinate [12% (w/v) in a solution of 0·9% (w/v) NaCl] (Hallett *et al.*, 1981; Hallett & Campbell 1983). Cells were suspended in Krebs–HEPES containing 120 mM NaCl, 4·8 mM KCl, 1·2 mM $MgSO_4$, 1·2 mM KH_2PO_4, 1·3 mM $CaCl_2$, 25 mM HEPES and 0·1% (w/v) bovine serum albumin, pH 7·4. The purification yielded approximately 10^8 cells from one rat, of which more than 98% were PMN as seen by phase contrast microscopy.

Titration of complement for lysis of rat PMN

PMN (1×10^7 cells) in Krebs–Hepes were antibody-sensitized by incubating in a 1:100 dilution of rabbit anti-rat liver 5′-nucleotidase for 5 min at 37°. The cells were washed and then further incubated at 37° in various dilutions of normal human serum (1:2 to 1:500). After 60 min, the cells were separated from the supernatant by centrifugation. The pellet was lysed in Nonidet P40 (3·5% v/v) and lactate dehydrogenase (LDH) activity at a final detergent concentration of 0·17% (v/v) was determined in the pellet and supernatant according to the method of Bergmeyer (1965). The percentage of cells lysed by complement was then assessed by the amount of lactate dehydrogenase released into the supernatant. The dilution of serum giving approximately 30–40% lysis was selected as the dose of complement in subsequent experiments.

Fluorescence measurements

PMN, stained with the membrane impermeable fluorescent nuclear indicator propidium iodide during complement lysis, were centrifuged for 2 seconds in the Eppendorf microcentrifuge. The cell pellet was washed and resuspended in Krebs–HEPES, and fluorescence measured in a Perkin-Elmer 204-A fluorescence spectrophotometer (excitation wavelength 534 nm, and emission wavelength 615 nm). Maximum possible fluorescence was determined for an equivalent number of PMN lysed by freeze-thawing and then stained with propidium iodide. The percentage of cells taking up propidium iodide during complement lysis was then estimated. Healthy cells were impermeable to propidium iodide, the fluorescence being less than 5% of maximum propidium iodide fluorescence of permeabilized cells.

Flow cytometry

Complement lysis of individual antibody-sensitized PMN was analysed with a Becton-Dickinson FACS III flow cytometer, fitted with a 50 μm nozzle and a thermostatically controlled sample housing. Distribution of red fluorescence emitted from cells stained with propidium iodide was detected through a 620 LP filter using an Argon laser with an excitation peak at 488 nm. The scatter distribution obtained simultaneously was accumulated in a multichannel (127 channel) pulse height analyser. Cells were counted and sorted at a flow rate of 2000 cells/second.

Characterization of cells isolated from the flow cytometer

Subpopulations of PMN after 30 min of complement attack were isolated from the cell sorter and washed. The cells, resuspended in Krebs–HEPES buffer containing 0·1% (w/v) bovine serum albumin, pH 7·4, were stored on ice for up to 30 min. Lactate dehydrogenase content of the cells was then assayed according to the method of Bergmeyer (1965). Cell viability was also determined by trypan blue exclusion (Hudson & Hay, 1980). The ability of cells to respond to stimuli was assessed by measuring the luminol-dependent chemiluminescence in response to latex beads (Hallett *et al.*, 1981). Results were expressed as a percentage of control cells.

Fluorescence microscopy

The fluorescence of PMN after sorting by flow cytometry was analysed with a Zeiss fluorescence microscope. Cells stained with propidium iodide were viewed using an excitation filter BP 546/12 and emission filter LP590. FITC-labelled monoclonal antibody MC9-36, recognizing MACs on PMN, was observed using excitation filter BP 450/490 and emission filter BP 520–560.

RESULTS

Propidium iodide as an indicator of cell permeability

Propidium iodide (MW 680) is a poorly fluorescent, water-soluble substance that becomes highly fluorescent when in contact with DNA. Healthy cells, impermeable to propidium iodide, therefore remain non-fluorescent. In contrast, isolated nuclei and cells made permeable to propidium iodide by the MAC became fluorescent within 1–2 seconds.

In order to use propidium iodide nuclear fluorescence to quantify increases in the permeability of individual cells, it was first necessary to characterize changes in fluorescence of the

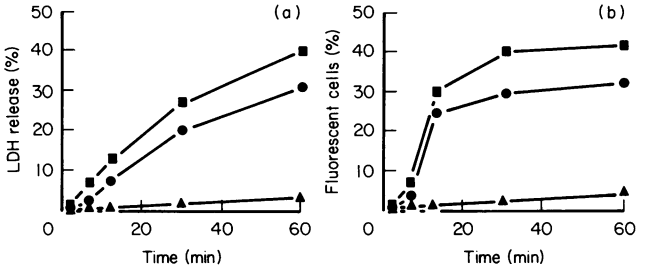

Figure 1. Antibody-sensitized PMNs (1×10^7 cell/ml) were incubated at 37° for 60 min with 1:40 final dilution of NHS. Aliquots were removed at intervals and cell lysis measured by (a) release of cytoplasmic LDH, and (b) permability to extracellular propidium iodide. Complement lysis was performed in the presence (■——■) and absence (●——●) of 10 μM 2-chloroadenosine. The experiment was repeated using PMNs not sensitized with antibody (▲——▲).

whole PMN population following formation of MACs. A suboptimal dose of complement was chosen causing approximately 30–40% lysis within 60 min as determined by lactate dehydrogenase release (Fig. 1a). Under these conditions, all cells (more than 95%) had MACs on the cell surface within 2–5 min of complement activation, as demonstrated visually by fluorescein-labelled monoclonal antibody to C9.

Formation of membrane attack complexes on the surface of PMN caused an increase in propidium iodide fluorescence after an initial lag of approximately 5–10 min, reaching an end-point of 30–40% of the total fluorescence within 30 min (Fig. 1b). In contrast, 1–2% lactate dehydrogenase release was detectable within 2 min of complement activation, but then increased in the extracellular fluid more slowly. The small early release of lactate dehydrogenase was not the result of cell permeability increase or lysis, but due to the release of vesicles formed via the protection mechanism (Morgan *et al.*, 1986). Lactate dehydrogenase release and nuclear propidium iodide fluorescence were less than 5% after 60 min incubation of non-antibody coated PMN with normal human serum as a source of complement. 2-Chloroadenosine is an inhibitor of the removal of MACs from the cell surface (Roberts *et al.*, 1985). At the end-point (30–60 min), propidium iodide fluorescence and lactate dehydrogenase release in the presence of 2-chloroadenosine was 10–15% greater than cells incubated without 2-chloroadenosine (Fig. 1a and b).

Measurement of permeability changes in individual rat PMN by flow cytometry

In order to interpret data obtained from population studies, and in particular to quantify the time taken to reach permeability and lytic thresholds in individual cells, PMN were analysed by flow cytometry. In the flow cytometer, single cells passed through a sensing zone where light scatter and propidium iodide fluorescence of individual cells were measured. The signal generated for 10,000 cells obtained within 5 seconds was displayed as a 'dot plot' (Fig. 2), where each dot represented light scatter and fluorescence emission of a single PMN. The signals were also used to produce a frequency distribution histogram of number of cells versus light scatter and fluorescence distribution.

The dot plot of the PMN incubated with propidium iodide after 30 min of complement attack revealed three distinct subpopulations of cells (Fig. 2) with (i) 'zero' fluorescence (equivalent to cellular autofluorescence) and high scatter (Population A), (ii) high fluorescence and high scatter (Population B), and (iii) high fluorescence and low scatter (Population C). A fourth subpopulation with medium fluorescence and low scatter (Population D) was detectable after 45 min of complement attack, and appeared to consist of fragmented nuclei with approximately half the fluorescence of intact (Population B) or lysed (Population C) cells.

Characterization of subpopulation of cells

Antibody-sensitized PMN after 30 min of complement attack were sorted by flow cytometry into Subpopulations A, B and C, and characterized morphologically by phase and fluorescence microscopy, and biochemically to determine whether the cells had lysed or were still intact, and whether they could still respond to cell stimuli. The latter was assessed using luminol-dependent chemiluminescence as an indicator of reactive oxygen metabolite production (Campbell, Hallett & Weeks, 1985). Cells with 'zero' fluorescence and high scatter (Population A) were morphologically intact, contained few or no detectable MACs on their surface, excluded trypan blue, contained 100% lactate dehydrogenase activity, and produced a luminol-dependent chemiluminescence similar to that of normal cells in response to a phagocytic stimulus. Since more than 95% of the cells had MACs on membranes after 5 min of complement attack, Population A consisted of cells with unstained nuclei,

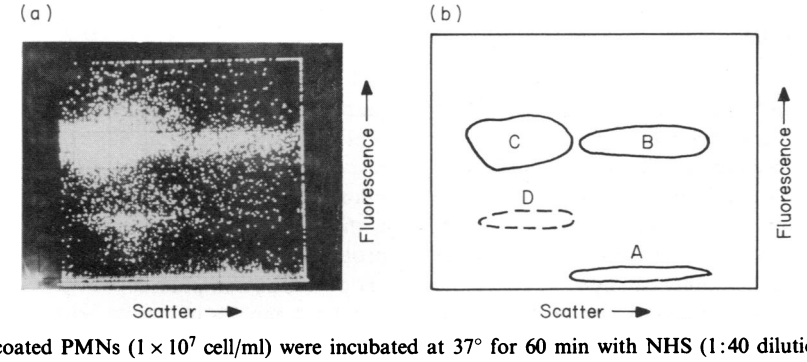

Figure 2. Antibody-coated PMNs (1×10^7 cell/ml) were incubated at 37° for 60 min with NHS (1:40 dilution). Analysis by flow cytometry (a) shows a 'dot plot' (scatter versus fluorescence plot), and a trace of the same plot (b) shows Subpopulations A, B and C representing cells with zero fluorescence/high scatter, high fluorescence/high scatter and high fluorescence/low scatter, respectively. Subpopulation D consisted of fragmented nuclei with half the fluorescence.

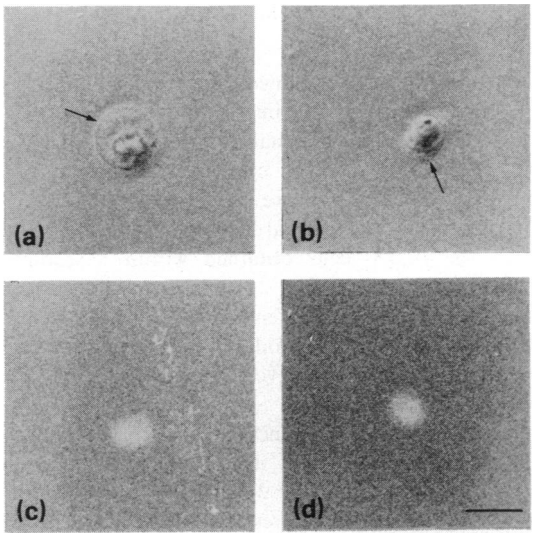

Figure 3. Antibody-sensitized PMNs after 30 min of complement attack at 37° were sorted by flow cytometry into subpopulations with (a and c) high fluorescence/high scatter (Population B), and (b and d) high fluorescence/low scatter (Population C). Cells were viewed under (a and b) phase contrast, and (c and d) fluorescence field. Note the presence of (a) intact, and (b) shrunken cell membrane, indicated by arrows. Calibration bar represents 10 μm.

which had protected themselves from complement attack without becoming permeable to propidium iodide.

Population B, with high light scatter, consisted of cells with fluorescent nuclei and intact cell membranes (Fig. 3), retaining more than 80% of their cytoplasmic lactate dehydrogenase and excluding trypan blue. The cells had thus become transiently permeable to propidium iodide prior to isolation, yet were not lysed and had recovered sufficiently from complement attack to produce a chemiluminescent response when challenged with latex beads. Like Population A, Population B bound little or no fluorescently labelled monoclonal antibody to C9, indicating a removal of membrane attack complexes from the cell surface.

Fluorescence microscopy of cells with low scatter (Population C) showed shrunken or lysed cells with fluorescent nuclei (Fig. 3). This population of cells retained less than 12% of their cytoplasmic lactate dehydrogenase, failed to exclude trypan blue, and produced no detectable chemiluminescent response: hence these cells were considered non-viable.

Time–course of propidium iodide fluorescence changes in subpopulations of PMN

The fluorescence distribution histogram of PMN attacked by the MAC showed that cells transformed from 'zero' fluorescence to high fluorescence very rapidly, their being apparently no intermediate-state cells. Within the intergral time for the 'dot plot' (5 seconds), the number of cells with fluorescence intermediate between zero and high fluorescence was 2·4%. Thus, the transition time from one state to another can be estimated to be less then 2·4% of 5 seconds (0·1 seconds). A plot of fluorescent cells (Populations B and C) against time showed that the first PMN became permeable to propidium iodide after an initial lag of $7{\cdot}5 \pm 2{\cdot}5$ min ($n=3$), whilst the remaining cells became permeable during the subsequent 50 min following complement attack (Fig. 4a). A similar lag was obtained before an increase in Population B could be detected. In contrast, cells in Population C were only detected after a lag of $11{\cdot}5 \pm 1{\cdot}0$ min ($n=3$). Hence the sequence for PMN attacked by complement involved the transformation of cells from Population A to Population B to Population C. Cells in Population A declined rapidly after the initial lag and reached end-point after 60 min of complement attack; however, end-point for Population C was reached between 30 min and 60 min, resulting in a continued accumulation of cells in Population B between 45 min and 60 min.

Effect of 2-chloroadenosine on individual PMN attacked by MACs

2-Chloroadenosine, a non-metabolizable analogue of adenosine, has been shown to act via A_2 receptors and intracellular cyclic AMP to inhibit removal of MAC and recovery of PMN from complement attack (Roberts *et al.*, 1985). 2-Chloroadenosine appeared to decrease the lag before propidium iodide fluorescent cells (Populations B and C) could be detected (Fig. 4a). This was clearly shown when the two populations of individual cells were quantified individually. Analysis of the changes in subpopulations of PMN attacked by MACs (Fig. 4b, c and d) showed that 2-chloroadenosine caused more cells in Population B to transform into Population C, resulting in an increase in Population C from 6% in controls to 17% with 2-chloroadenosine after 60 min. In contrast, cells accumulating in Population B declined from 20% in controls to 13% after 60 min with 2-chloroadenosine.

DISCUSSION

Flow cytometry provides a powerful analytical tool for studying the time course of permeability changes in individual cells attacked by the MAC of complement. The results reported here show that both the time course and the end-point permeability of a population of PMNs to small molecules (MW approximately 500–1000) and to macromolecules (Fig. 1) is explained by different cells reaching the threshold for permeability at different times after formation of the MAC (Fig. 4). They further support our previous reports that PMNs can protect themselves from attack by complement (Campbell & Morgan, 1985; Morgan & Campbell, 1985) and eventually recover (Morgan *et al.*, 1987). Thus, the end-point in a population of cells permeabilized to small molecules, or lysed, depends on the rate of removal of MACs relative to the time taken to reach the two distinct threshold points in each well.

Three subpopulations of cells were identified and quantified in the flow cytometer (Figs 2 and 4), and then characterized morphologically and biochemically. Under the conditions of these experiments, some 60% of cells remained intact, able to respond to stimuli, and did not show propidium iodide nuclear fluorescence, yet more than 95% of the cells contained MACs within 2–5 min of complement activation. This implied that the protection mechanism, removing the MAC via budding or endocytosis (Campbell & Morgan, 1985; Morgan *et al.*, 1987), can be complete not only before cell death but also before permeabilization to small molecules. The time-sequence of events in individual cells attacked by complement thus seems to involve an increase in cytoplasmic free Ca^{2+} in the range 1–30 μM within 5 seconds of C9 binding to the membrane (Campbell

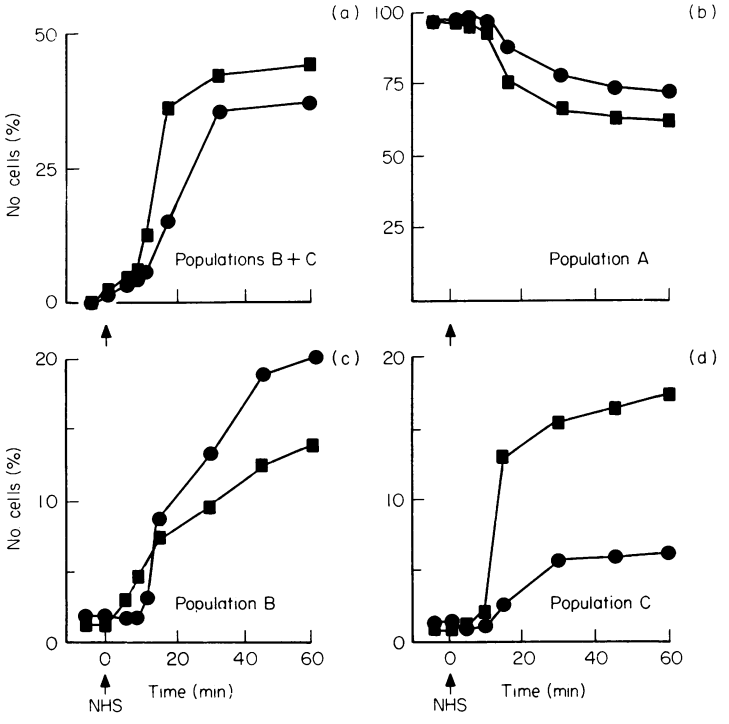

Figure 4. Antibody-sensitized PMNs (1×10^7 cells/ml) incubated at 37° with NHS (1:40 dilution) were analysed by flow cytometry. Windows were selected to allow quantification of subpopulations of PMNs with (a) high fluorescence (Populations B and C), (b) zero fluorescence/high scatter (Population A), (c) high fluorescence/high scatter (Population B), and (d) high fluorescence/low scatter (Population C). Experiments were performed in the presence (■——■) and absence (●——●) of 10 μM 2-chloroadenosine. The values quoted are expressed as a percentage of 10,000 cells analysed, and are plotted against time after the addition of complement.

et al., 1981). This, together with mechanisms independent of a rise in intracellular Ca^{2+} (Morgan & Campbell, 1985), activates a protection mechanism that can remove all of the MACs from some cells within 2–5 min. The first cells to show an increase in permeability to small molecules were detected within 5 min of complement attack. However, some cells did not show such an increase in permeability until 30–45 min after formation of MAC on cell surfaces. The threshold for cell death detected using flow cytometry occurred at least 2·5 min after the permeability threshold to small molecules. One problem that needs to be resolved is a lack of correlation between total lactate dehydrogenase and lysis of individual cells (Fig. 4a). This suggests that some lactate dehydrogenase can be released from cells not irreversibly damaged by complement, and in these circumstances is not a good quantitative criterion of cell death.

The question now arises how this time sequence describing the cell biology of the MAC can be related to its molecular biology (Stanley *et al.*, 1986). It is not yet known whether the rise in intracellular Ca^{2+} occurs simply on C9 binding to the membrane or whether C9 insertion is required. Nor is the significance of C9 polymerization clear, particularly since lysis of cells occurs in the absence of polymerization (Dankert & Esser, 1985). The molecular basis of the three permeability thresholds, namely Ca^{2+}, small molecules (MW 500–1000) and macromolecules, is intriguing. These could involve thresholds in C9 in each cell, for example binding, insertion, aggregation, or polymerization, or thresholds in the cell itself, for example a rapid rise in intracellular Ca^{2+} concentration, vesiculation or gel-sol transformation in the cytoplasm. The latter process is known to be regulated by the Ca^{2+} binding proteins (Weeds, 1982) found in many cells, including PMNs.

Inhibition of the protection mechanism increases the number of cells permeabilized to small molecules, and increases the number of cells eventually lysed. 2-Chloroadenosine (Roberts *et al.*, 1985) thus increased the number of fluorescent cells and lysed cells (Fig. 4a). Analysis of the time sequence (Fig. 4b, c and d) showed that 2-chloroadenosine also reduced the lag-time before fluorescent cells could be detected. This suggested that 2-chloroadenosine, acting via cyclic AMP, not only reduced the rate of MAC removal in PMNs but also reduced the time to the threshold for permeability to small molecules.

It has been known for more than 25 years that nucleated cells are relatively resistant to complement attack (Green & Goldberg, 1960). Two mechanisms of resistance have now been identified: firstly prevention of MAC formation, and secondly an active protection mechanism involving a removal of MAC from cell membranes. Cyclic AMP may activate (Boyle, Ohanian & Borsos, 1976) or inhibit (Roberts *et al.*, 1985) these latter processes, depending on the cell type. The characterization of the molecular basis of these protection mechanisms together with that of the thresholds responsible for permeability changes and activation of individual cells (Campbell, 1983, 1987) is likely to lead to new insights into the biological role of the terminal complement component C9. The similarities between the structure and cellular effects of C9 compared with lymphocyte perforin (Lachmann, 1983; Young *et al.*, 1986) and bacterial toxins (Bhakdi *et al.*, 1985) suggest that permeability thresholds and recovery mechanisms also exist with these other membrane-

damaging agents. The conceptual and experimental approach described in this paper thus provides a basis for a better understanding of the role of these molecular complexes in the pathogenesis of immune-based diseases.

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REFERENCES

BERGMEYER M.U. (1965) *Methods of Enzymatic analysis* (ed. M. U. Bergmeyer), p. 574. Academic Press, New York.

BHAKDI S., SEEGER W., SUTTORP N. & TRANUM-JENSEN T. (1985) Damage to the host by channel-forming bacterial toxins. In: *The Pathogenesis of Bacterial Infections*, p. 268. Springer Verlag, Berlin.

BIESECKER G. (1983) Biology of disease. Membrane attack complex of complement as a pathologic mediator. *Lab. Invest.* **49,** 237.

BOYLE M.D.P., OHANIAN S.H. & BORSOS T. (1976) Studies on the terminal stages of antibody-mediated killing of a tumour cell. *J. Immunol.* **116,** 1276.

CAMPBELL A.K. (1983) *Intracellular Ca^{2+}: Its Universal Role as Regulator*. John Wiley, Chichester, U.K.

CAMPBELL A.K. (1987) Intracellular calcium: friend or foe. *Clin. Sci.* **125** (in press).

CAMPBELL A.K., DAW R.A., HALLETT M.B. & LUZIO J.P. (1981) Direct measurement of the increase in intracellular free calcium ion concentration in response to the action of complement. *Biochem. J.* **194,** 551.

CAMPBELL A.K., HALLETT M.B. & WEEKS I. (1985) Chemiluminescence as an analytical tool in cell biology and medicine. *Meth. Biochem. Analyt.* **31,** 317.

CAMPBELL A.K. & LUZIO J.P. (1981) Intracellular free calcium as a pathogen in cell damage initiated by the immune system. *Experientia*, **37,** 110.

CAMPBELL A.K. & MORGAN B.P. (1985) Monoclonal antibodies demonstrate protection of polymorphonuclear leukocytes against complement attack. *Nature (Lond.)*, **317,** 164.

DANKERT J.R. & ESSER A.F. (1985) Proteolytic modification of human complement protein C9. *Proc. natl. Acad. Sci. U.S.A.* **82,** 2128.

EDWARDS S.W., MORGAN B.P., HOY T.G., LUZIO J.P. & CAMPBELL A.K. (1983) Complement-mediated lysis of pigeon erythrocyte ghost analysed by flow cytometry. Evidence for the involvement of a 'threshold' phenonemon. *Biochem. J.* **216,** 195.

GREEN H. & GOLDBERG B. (1960) The action of antibody and complement on mammalian cells. *Ann. NY Acad. Sci.* **87,** 352.

HALLETT M.B. & CAMPBELL A.K. (1983) Two distinct mechanisms for stimulation of oxygen-radical production by polymorphonuclear leucocytes. *Biochem J.* **216,** 459.

HALLETT M.B., LUZIO J.P. & CAMPBELL A.K. (1981) Stimulation of Ca^{2+}-dependent chemiluminescence in rat polymorphonuclear leucocytes by polystyrene beads and non-lytic action of complement. *Immunology*, **44,** 569.

HUDSON L. & HAY F.C. (1980) *Practical Immunology* (eds L. Hudson and F. C. Hay), p. 29. Blackwell Scientific, Oxford.

LACHMANN P.J. (1983) Are complement lysis and lymphocytotoxicity analogous? *Nature (Lond.)*, **305,** 473.

MAYER M.M., MICHAEL D.W., RAMM L.E., WHITLOW M.B., WILLOUGHBY S.B. & SHIN M.L. (1981) Membrane damage by complement. *Crit. Rev. Immunol.* **2,** 133.

MORGAN B.P. & CAMPBELL (1985) The recovery of human polymorphonuclear leucocytes from sublytic complement attack is mediated by changes in intracellular free calcium. *Biochem. J.* **231,** 205.

MORGAN B.P., CAMPBELL A.K., LUZIO J.P. & HALLETT M.B. (1984a) Recovery of polymorphonuclear leucocytes from complement attack. *Biochem. Soc. Trans.* **12,** 779.

MORGAN B.P., DANKERT J.R. & ESSER A.F. (1987) Recovery of human neutrophils from complement attack: removal of the membrane attack complex by endocytosis and exocytosis. *J. Immunol.* (in press).

MORGAN B.P., DAW R.A., SIDDLE K., LUZIO J.P. & CAMPBELL A.K. (1983) Immunoaffinity purification of human complement component C9 using monoclonal antibodies. *J. immun. Meth.* **64,** 269.

MORGAN B.P., SEWRY C.A., SIDDLE K., LUZIO J.P. & CAMPBELL A.K. (1984b) Immunolocalization of complement component C9 on necrotic and non-necrotic muscle fibres in myositis using monoclonal antibodies: a primary role of complement in autoimmune cell damage. *Immunology*, **52,** 181.

MULLER-EBERHARD H.J. (1984) The membrane attack complex. *Springer Seminar Immunopath.* **7,** 93.

PODACK E.R., TSCHOPP J. & MULLER-EBERHARD H.J. (1982) Molecular organisation of C9 within the membrane attack complex of complement. Induction of circular C9 polymerisation by the C5b–8 assembly. *J. exp. Med.* **156,** 268.

ROBERTS P.A., MORGAN B.P. & CAMPBELL A.K. (1985) 2-chloroadenosine inhibits complement-induced reactive oxygen metabolite production and recovery of human polymorphonuclear leucocytes attacked by complement. *Biochem. biophys. Res. Common.* **126,** 692.

STANLEY K.K., EDWARDS M.R. & LUZIO J.P. (1980) Subcellular distribution and movement of 5'-nucleotidase in rat cells. *Biochem. J.* **186,** 59.

STANLEY K.K., PAGE M., CAMPBELL A.K. & LUZIO J.P. (1986) A mechanism for the insertion of complement component C9 into target membranes. *Molec. Immunol.* **23,** 451.

TSCHOPP J., ENGEL A. & PODACK E.R. (1984) Molecular weight of Poly (C9). *J. Biol.* **259,** 1922.

WEEDS A. (1982) Actin-binding protein-regulators of cell architecture and motility. *Nature (Lond.)*, **296,** 811.

YOUNG J.D.-E., HENGARTNER H., PODACK E.R. & COHN Z.A. (1986) Purification and characterization of a cytolytic pore-forming protein from granules of cloned lymphocytes with natural killer activity. *Cell*, **44,** 849.