The recovery of human polymorphonuclear leucocytes from sublytic complement attack is mediated by changes in intracellular free calcium

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Using polymorphonuclear leucocyte–erythrocyte ghost hybrids entrapping the calcium-activated photoprotein obelin, we have demonstrated that sublytic amounts of the complement membrane attack complex induce a rapid but transient increase in intracellular free calcium ion concentration ($[Ca^{2+}]_i$). This increase in $[Ca^{2+}]_i$ occurs prior to, and is required for, rapid removal of membrane attack complexes from the cell surface. The increase in $[Ca^{2+}]_i$ is not only due to increased influx from outside the cell, but also results from mobilization of intracellular stores. The possible mechanism of mobilization of calcium, and the importance of an increase in $[Ca^{2+}]_i$ as a mediator of recovery processes in nucleated cells, are discussed.

INTRODUCTION

Nucleated cells are much more resistant to complement lysis than are erythrocytes (Ohanian & Schlager, 1981). Our studies have been aimed at establishing the mechanisms responsible for resistance. We have demonstrated in PMN that resistance to complement lysis occurs because of removal of MACs from the cell surface (Morgan *et al.*, 1984a; Roberts *et al.*, 1985). These findings have recently been confirmed in another cell type, the Ehrlich cell (Carney *et al.*, 1985). We have also previously shown that a very early event following formation of the MAC is a rapid increase in intracellular free calcium ion concentration ($[Ca^{2+}]_i$) occurring within about 5 s of binding of C9 (Campbell *et al.*, 1979, 1981), and have established that this can activate processes in nucleated cells (Campbell & Hallett, 1983; Morgan, 1984). The purpose of this study was to investigate whether this increase in $[Ca^{2+}]_i$ was also responsible for mediating removal of MACs resulting in recovery of PMN from complement attack.

In order to correlate $[Ca^{2+}]_i$ with recovery processes, the calcium-activated photoprotein obelin was introduced into PMN by hybridization with erythrocyte ghosts entrapping obelin (Campbell & Hallett, 1983). We show here that the rapid disappearance of MACs from the surface of PMN–erythrocyte ghost hybrids exposed to sublytic complement attack is dependent upon an initial complement-induced increase in $[Ca^{2+}]_i$.

MATERIALS AND METHODS

Cells

Human PMN were prepared by a modification of published methods (Skoog & Beck, 1956). Briefly, 50 ml of fresh blood was drawn into 10 ml of acid citrate dextrose (ACD). The blood was then mixed with 30 ml of 6% dextran 70 in 0.9% saline in a tall plastic cylinder, and allowed to settle for 60 min. The supernatant was

carefully removed and centrifuged at 1000 *g* for 3 min to pellet the cells. Residual erythrocytes were lysed by suspending the cell pellet in 5 ml of water for 30 s. Iso-osmoticity was restored by addition of 5 ml of 1.8% NaCl, and the cells were again pelleted and resuspended in 10 ml of Krebs–Hepes buffer, pH 7.4 (120 mM-NaCl, 4.8 mM-KCl, 1.2 mM-KH₂PO₄, 1.2 mM-MgSO₄, 1.3 mM-CaCl₂, 25 mM-Hepes, 0.1% bovine serum albumin). Ficoll-Paque (Pharmacia) (10 ml) was then injected under the cell suspension which was centrifuged at 1000 *g* for 30 min. The cell pellet, containing the PMN, was washed twice in Krebs–Hepes buffer by centrifugation and resuspension as before. Yield from 50 ml of blood was 5×10^7 – 7×10^7 cells, of which more than 95% were viable PMN.

Human erythrocyte ghosts containing obelin plus or minus EGTA were prepared from fresh human erythrocytes, as previously described (Campbell & Hallett, 1983). Entrapment of obelin was estimated by measuring luminescence of ghosts lysed by detergent. Entrapment of EGTA was estimated using trace amounts of [¹⁴C]EDTA.

Fusion of PMN and erythrocyte ghosts

PMN and erythrocyte ghosts were fused as previously described (Campbell *et al.*, 1981). Briefly, a mixture of PMN (5×10^7 cells) and erythrocyte ghosts (5×10^7 cells) were suspended in 200 μ l of calcium-free fusion medium (150 mM-NaCl, 10 mM-Tes/Chelex, 0.25 mM-uranyl acetate, pH 7.4) containing u.v.-inactivated Sendai virus. The mixture was incubated at 0 °C for 10 min, then at 37 °C for 30 min. Unfused PMN were separated from hybrids by centrifugation through a preformed Percoll gradient (1.06–1.12 g/ml) at 1000 *g* for 30 min. Yield of hybrids was about 50%, and viability was > 95% by Trpan Blue exclusion. Viability has been confirmed by comparison of oxygen radical production, ATP and lactate dehydrogenase content, and lactate dehydrogenase release rate in hybrids and unhybridized PMN (Campbell & Hallett, 1983).

Abbreviations used: MAC(s), membrane attack complex(es); PMN, polymorphonuclear leucocytes; C, complement.

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Production of hybrids bearing sublytic amounts of the MAC

Hybrid cells bearing sublytic amounts of the MAC (HAC1-9 cells) were made as previously described for unhybridized PMN (Roberts *et al.*, 1985). Briefly, hybrids sensitized by incubation with an IgM monoclonal antibody against human PMN were first incubated at 37 °C for 15 min with various dilutions of C9-depleted serum. The cells were pelleted, washed and resuspended in ice-cold Krebs-Hepes buffer. These HAC1-8 cell intermediates were then incubated for 15 min at 0 °C with an excess of C9 to form HAC1-9 cells. After washing to remove unbound C9, these cells were incubated at 37 °C for 90 min, and end-point lysis was measured by Trypan Blue exclusion. The minimum dilution of C9-depleted serum that yielded HAC1-8 cell intermediates that after incubation with C9 at 0 °C did not significantly lyse (specific lysis < 5%) on subsequent incubation at 37 °C was used to produce sublytic HAC1-9 cells for the following experiments. Sublytic HAC1-9 cells were stable on storage at 0 °C for at least 4 h, but were always used within 2 h of production to ensure consistent results.

Determination of MACs on hybrids

Sublytic HAC1-9 cells (10^7 cells in 2 ml of Krebs-Hepes buffer) were incubated at 37 °C, and 0.2 ml portions were removed at intervals and placed on ice. Radiolabelled monoclonal antibody against C9, MC47 (Morgan *et al.*, 1983) (10^5 d.p.m., 3 ng) was added and incubation was continued on ice for 15 min. The cells were washed three times by centrifugation and resuspension in ice-cold Krebs-Hepes buffer, cell number and viability were estimated, and radioactivity in the washed cell pellet was measured. HAC1-8 cell intermediates treated similarly were used as controls.

To examine the calcium dependence of 'recovery' processes, the above experiment was repeated using: (a) HAC1-9 cells suspended in calcium-free Krebs-Hepes buffer containing 1 mM-EGTA, and (b) HAC1-9 cells suspended in calcium-free Krebs-Hepes buffer containing 1 mM-EGTA, and entrapping EGTA at a concentration of 0.5 mM.

Measurement of intracellular free calcium ion concentration during sublytic complement attack

A major advantage of using the photoprotein obelin to measure $[Ca^{2+}]_i$ is that its large size (M_r 30000) makes it much less likely to leak out of the complement-damaged cell than the smaller calcium-specific fluorophores (e.g. quin2). Sublytic HAC1-9 cells with obelin entrapped (2×10^6 cells in 200 μ l of ice-cold Krebs-Hepes buffer) were injected into a plastic tube containing 800 μ l of Krebs-Hepes buffer at 48 °C (to bring the cells immediately to 37 °C) in the same housing of a specially constructed digital luminometer. Obelin luminescence was monitored, and $[Ca^{2+}]_i$ was estimated at several time-points by measurement of the rate constant (photons $\cdot$ s $^{-1}$ /active photoprotein) at each time-point as previously described (Campbell *et al.*, 1985).

The above experiment was again repeated using: (a) HAC1-9 cells suspended in calcium-free Krebs-Hepes buffer containing 1 mM-EGTA, and (b) HAC1-9 cells suspended in calcium-free Krebs-Hepes buffer containing

Fig. 1. Loss of detectable MACs from PMN-erythrocyte ghost hybrids monitored by binding of anti-C9 antibody

Hybrids bearing sublytic amounts of MACs (HAC1-9 cells), produced as described in the Materials and methods section, were incubated at 37 °C, and the relative amount of MACs remaining on the cell surface was estimated by measuring specific binding of radiolabelled monoclonal antibody against C9. The results are expressed as a percentage of the specific antibody binding to HAC1-9 cells prior to incubation at 37 °C. Each point represents the mean value at that time in three separate experiments. Error bars indicate the standard deviation of the three determinations. Extracellular calcium 1.3 mM, no intracellular chelator (●); no extracellular calcium (1 mM-EGTA), no intracellular chelator (○); no extracellular calcium (1 mM-EGTA), intracellular EGTA, 0.5 mM (□).

1 mM-EGTA, and entrapping EGTA at a concentration of about 0.5 mM.

RESULTS

Loss of MACs from PMN-erythrocyte ghost hybrids under sublytic complement attack

Monoclonal antibody MC47 raised against native C9 had previously been shown to bind to C9 after its incorporation into the MAC on target cells (Morgan *et al.*, 1984b). This antibody was thus chosen to detect and quantify MACs on the hybrid cells in the present study. Incubation of sublytic HAC1-9 cells at 37 °C in Krebs-Hepes buffer caused a rapid decrease in uptake of radiolabelled MC47 antibody, indicating a loss of detectable MACs from the cell surface (Fig. 1). The half-time of disappearance of MACs was approx. 4 min. Lysis at end-point was 5%.

Removal of extracellular calcium inhibited the loss of MACs from the cell surface (Fig. 1). The half-time of disappearance was increased to about 7 min. Concomitantly, end-point lysis was increased 2-3-fold.

Entrapment of EGTA (0.5 mM) within the hybrids further inhibited the loss of MACs from the cell surface (Fig. 1). The half-time of disappearance of MACs was increased to about 25 min, and lysis at end-point was increased 5-fold. The total number of cells (lysed plus unlysed) recovered after incubation and washing did not differ significantly between the three experimental conditions used.

Fig. 2. Increase in intracellular free calcium concentration induced by the MAC

Hybrids bearing sublytic amounts of the MAC, and containing the calcium-activated photoprotein obelin plus or minus EGTA, were incubated at 37 °C in a luminometer. Intracellular free calcium ion concentration ($[Ca^{2+}]_i$) was calculated from the measured obelin luminescence as described in the Materials and methods section. Each point represents the average calculated $[Ca^{2+}]_i$ at that time in three separate experiments; error bars indicate the s.d. of these determinations. Extracellular calcium 1.3 mM, no intracellular chelator (●); no extracellular calcium (1 mM-EGTA), no intracellular chelator (○); no extracellular calcium (1 mM-EGTA), intracellular EGTA 0.5 mM (□).

Sublytic complement attack causes an increase in intracellular free calcium ion concentration

Placing sublytic HAC1-9 cells in calcium-containing Krebs-Hepes buffer at 37 °C caused an immediate increase in obelin luminescence. Estimated $[Ca^{2+}]_i$ rose from a basal level (measured in HAC1-8 cell intermediates) of 0.2 μ M to a peak of about 3 μ M within 60 s of commencing incubation (Fig. 2) and then fell gradually, reaching 1.4 μ M by 180 s. Measurements were not possible beyond this time, as all entrapped obelin was consumed. Removal of extracellular calcium slowed, but did not abolish, the increase in obelin luminescence. The estimated $[Ca^{2+}]_i$, after an initial lag of about 50 s, rose slowly to reach a peak of 1.5 μ M after a further 110 s (Fig. 2), and then fell slowly. Entrapment of EGTA (0.5 mM) within hybrids abolished the increase in obelin luminescence when HAC1-9 cells suspended in calcium-free medium were incubated at 37 °C, indicating that $[Ca^{2+}]_i$ did not significantly increase over the time course of the experiment (Fig. 2).

DISCUSSION

Using PMN-erythrocyte ghost hybrids we demonstrate here that sublytic amounts of MAC can induce a rapid, transient increase in $[Ca^{2+}]_i$. The increase in $[Ca^{2+}]_i$ begins prior to detectable removal of MACs from the cell surface. In the absence of extracellular calcium the increase in $[Ca^{2+}]_i$ was delayed and inhibited, but not

abolished. Concomitantly, the rate of removal of MACs from the cell surface was slowed. Intracellular chelating agents further inhibited the increase in $[Ca^{2+}]_i$, and greatly slowed removal of MACs from the cell surface.

These results provide compelling evidence that an increase in $[Ca^{2+}]_i$ is a mediator of cell recovery following sublytic complement attack. Inhibition of recovery processes by slowing or abolishing the increase in $[Ca^{2+}]_i$ resulted in increased cell death; up to 5-fold in these experiments. This is consistent with the concept of a critical threshold, whereby prolonging the time each cell takes to recover increases the probability that its lytic threshold will be reached. We had previously demonstrated in pigeon erythrocyte ghosts that the rapid increase in $[Ca^{2+}]_i$ upon formation of the MAC was caused by an influx of calcium, and was lost in the absence of extracellular calcium (Campbell *et al.*, 1981). In the PMN-erythrocyte ghost hybrid, however, it is clear that the MAC-induced increase in $[Ca^{2+}]_i$ is a result not only of influx, but also of release of calcium from intracellular stores. The latter process is evident in the absence of extracellular calcium, and commences after a short lag. The mechanism of release from intracellular stores remains to be established. However, it has recently been suggested that the MAC can activate specific membrane phospholipases (Imagawa *et al.*, 1983; Betz & Hansch, 1984), and activation of phospholipase C results in production of inositol trisphosphate which has been demonstrated to signal release of calcium from intracellular stores (Berridge & Irvine, 1984). It is thus tempting to speculate a mechanism whereby the MAC activates phospholipase C, resulting in the release of calcium from intracellular stores.

It has been demonstrated that $[Ca^{2+}]_i$ is a regulator of many cellular processes (reviewed in Campbell, 1983). Thus the MAC-induced increase in $[Ca^{2+}]_i$, as well as mediating recovery processes in nucleated cells, may also cause metabolic changes in the cell in the absence of lysis. These non-lytic effects may be of considerable importance in the pathogenesis of immune-mediated diseases.

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