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# REVIEW ARTICLE

# Complement membrane attack on nucleated cells: resistance, recovery and non-lethal effects

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## INTRODUCTION

It is now 100 years since the heat-labile bactericidal property of serum we now know as complement was first described (Nuttal, 1888; Buchner, 1889). Buchner called this labile serum factor 'alexin', from the Greek meaning 'without a name'. Later studies by Bordet (1896, 1898) and by Ehrlich (Ehrlich & Morgenroth, 1899, 1900) demonstrated that this same factor was involved in the lysis of erythrocytes by immune serum. Ehrlich coined the term 'complement' to indicate his belief that this factor augmented or 'complemented' the bacterolytic and haemolytic activity inherent in antibody. Now, a century on, we know that the complement system consists of a complex group of over 25 proteins present in plasma or on cell membranes which interact in a precise and tightly controlled manner to bring about a diverse range of effects which include the stimulation of inflammation, enhancement of phagocytosis, and cytolysis. This last effect is mediated by the terminal five components of the complement system, C5b–C9. These fluid-phase proteins possess the unique ability to self-associate after complement activation to form a membrane-associated heteropolymer, the membrane attack complex (MAC). The structure and mode of action of the MAC has stimulated intense debate over the last 20 years. The elegant electron-microscopic demonstration by Humphrey and co-workers (Borsos *et al.*, 1964) of ring-like lesions in complement-lysed membranes led Mayer to propose that the five terminal complement components self-associate to form a ring with a water-filled central pore which penetrates the membrane, thereby causing cytolysis (Mayer, 1972). Others suggested that the MAC functioned by disrupting membrane lipid around its site of insertion, thus creating a leaky patch rather than a rigid pore (Esser *et al.*, 1979). In the last few years it has been convincingly demonstrated that the major part of the ring lesion seen on complement-lysed membranes is composed of up to 16 C9 molecules arranged in a rigid ring structure, the other terminal components, with the possible exception of the α-chain of C8, occupying a peripheral position in the lesion (Podack, 1988). This structure would appear to support Mayer's hyothesis, albeit with a different molecular composition of the ring itself. However, several lines of evidence suggest that this rigid lesion is not necessary for the expression of MAC effects: C9 which has been proteolytically modified to prevent ring polymer formation is fully lytically active (Dankert & Esser, 1985; Morgan *et al.*, 1987*b*); MACs containing only a single C9 molecule per complex cause efficient lysis of erythrocytes (Bhakdi & Tranum-Jensen, 1986); functional studies indicate that only four C9 molecules per C5b–8 complex are required to form a lesion through which large molecules can pass (Ramm *et al.*, 1984); and immunochemical studies show that an average of only three or four C9 molecules are bound per C5b–8 complex on complement-lysed cells (Sims, 1983; Stewart *et al.*, 1984).

Although, as indicated above, MAC structure has been and remains an area of controversy, the mechanisms by which the MAC damages cells have attracted much less attention. This has stemmed in large part from the ubiquitous use of the aged sheep erythrocyte as a target for studying lysis by the MAC. This metabolically inert target is extremely sensitive to lysis, a single functional lesion being sufficient to initiate cell swelling and osmotic lysis (Mayer, 1972). More subtle cell-damaging effects of the MAC would therefore not be detected in this model, resulting in the popular view of the MAC as a moiety which rapidly and efficiently kills cells (Roitt, 1984). The purpose of this review is to dispel these misconceptions by describing the effects of complement membrane attack on metabolically active nucleated cells. The mechanisms by which these cells escape lysis by the MAC and the effects of non-lethal attack will be described, and a role for these events in the pathogenesis of inflammatory diseases postulated.

## NUCLEATED CELLS RESIST LYSIS BY COMPLEMENT

Nucleated cells are more difficult to kill with antibody and complement than are metabolically inert targets like aged erythrocytes or liposomes. As an example of this fact, Fig. 1 compares the killing of optimally antibody-sensitized human neutrophils by homologous and heterologous complement with killing of aged human erythrocytes. With both sera lysis of erythrocyte is complete at doses which cause no detectable killing of neutrophils. This section will detail the phenomenon of nucleated cell resistance and the cellular mechanisms involved.

### Background

Although the effects of complement and antibody on nucleated cells in culture have been studied since the beginning of this century (Lambert & Hanes, 1911), clear documentation of the effects at the cellular level did not occur until the 1950's (Kalfayan & Kidd 1953; Miller & Hsu, 1956; Ellem, 1957, 1958; Green & Goldberg, 1960). Goldberg and Green demonstrated that attack on Krebs

Abbreviations used: MAC, membrane attack complex; $[Ca^{2+}]_i$, intracellular free calcium ion concentration; HETE, hydroxyeicosatetraenoic acid; TCC, terminal complement complex.

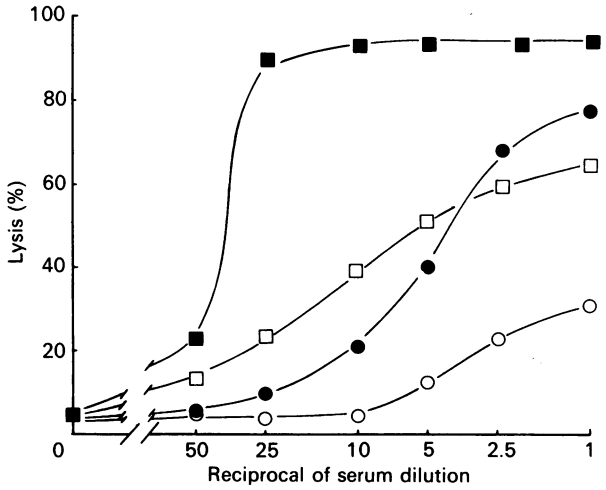


**Fig. 1. Lysis of human neutrophils and erythrocytes by homologous and heterologous complement**

Neutrophils and erythrocytes were optimally sensitized using appropriate antibodies and then incubated at 37 °C with varying dilutions of human serum (□, ○) or guinea pig serum (■, ●) for 90 min. Neutrophil lysis was estimated by measuring release of lactate dehydrogenase (○, ●), and erythrocyte lysis by release of haemoglobin (□, ■).

ascites tumour cells by complement differed from erythrocyte killing in several important respects. The cells became swollen and released intracellular amino acids, potassium and RNA, but maintained a grossly intact cell membrane even after prolonged exposure to complement (Goldberg & Green, 1959; Green *et al.*, 1959). This relative resistance of nucleated cells to lysis by complement was initially ascribed to low concentrations of antigen on the cell surface (Moller & Moller, 1962; Linscott, 1970). However, others have subsequently demonstrated no correlation between antigen density and mobility, or the amount of C1 bound and cell susceptibility to lysis (Pellegrino *et al.*, 1974; Boyle *et al.*, 1975; Lerner *et al.*, 1971; Segerling *et al.*, 1976). In 1970, Cikes made the important observation that the sensitivity of continuously growing virus-transformed murine lymphoma cells to killing by antibody and complement varies with the phase of the cell cycle (Cikes, 1970*a*,*b*). The cells were most sensitive in the stationary phase and almost completely insensitive in the exponential phase (Cikes & Freiberg, 1971). These findings were confirmed in other cell types (Shipley, 1971; Pasternak *et al.*, 1971; Ferrone *et al.*, 1973; Pellegrino *et al.* 1974; Ohanian *et al.*, 1983), though the stage of the cycle at which cells were most sensitive differed from cell to cell. The variation in sensitivity was shown to be unrelated to the number of molecules of early components (C4 and C3; Ohanian & Borsos, 1975) or late components (C5 and C8; Cooper *et al.*, 1974) bound per cell. It was thus apparent that activation of the complement system and MAC formation on the surface of a nucleated cell did not inevitably lead to cell lysis, the final outcome being influenced by the metabolic state of the cell.

## Metabolic factors

What then are the important metabolic parameters which influence whether a cell is lysed by the MAC or not? In a comprehensive series of studies, Ohanian, Borsos and co-workers examined the effects of pretreatment with a wide range of chemical and physical agents on the outcome of complement attack on sensitive and resistant guinea pig hepatoma cell lines (Segerling *et al.*, 1974; Schlager & Ohanian, 1977, 1978, 1980; Schlager *et al.*, 1978*a*,*b*). Their results, summarized in Table 1, showed that treatment of these cells with inhibitors of cellular metabolism, notably chemotherapeutic drugs enhanced their susceptibility to lysis by complement. These results were confirmed by others on different cell types (Ferrone *et al.*, 1973; Liang & Cohen, 1975). However, the mechanisms of this enhancement were unclear because the concentrations of inhibitor and time of treatment required were far greater than was necessary for inhibition of synthesis of DNA, RNA, protein or complex carbohydrate, and other inhibitors of synthesis of these macromolecules, such as sodium azide and sodium cyanide, caused no enhancement of lytic susceptibility. Inhibitors of contractile microfilament function did not, at least in the short term, influence the sensitivity of the cells to complement lysis, but inhibitors of complex lipid synthesis markedly enhanced cell killing, an effect which was reversible upon removal of the drug, suggesting that lipid synthesis was important in cell

**Table 1. Enhancers and inhibitors of nucleated cell killing**

Cells were exposed to the agents prior to attack with antibody and complement. Cell lysis was then compared with controls which had not been pretreated. Enhancement of lysis (at least 2-fold) (+); inhibition of lysis (−), no effect (0). Modified from Ohanian & Schlager (1981).

| Agent | Effect | Comment |
|---|---|---|
| Metabolic inhibitors and chemotherapeutic drugs | | |
| Actinomycin D | + | Very high concentrations of drugs; temperature-dependent; reversible; variable with different cell lines |
| Adriamycin | + | |
| Puromycin | + | |
| 5-Fluorouracil | 0 | |
| Cytosine arabinoside | 0 | |
| Hydroxyurea | 0 | |
| Cyclophosphamide | 0 | |
| Vincristine | 0 | |
| $NaN_3$ | 0 | |
| NaCN | 0 | |
| Colchicine | 0 | |
| Cytochalasin B | 0 | |
| Enzymes | | |
| Neuraminidase | + | Reversibility dependent on protein synthesis |
| Trypsin | + | |
| Pepsin | + | |
| Pronase | + | |
| Elastase | + | |
| DNAase | 0 | |
| RNAase | 0 | |
| Lipase | 0 | |
| Hormones | | |
| Insulin | − | Inhibitory effects transient even in continued presence of hormone |
| Hydrocortisone | − | |
| Prednisone | − | |
| Adrenaline | − | |
| Physical agents | | |
| Hyperthermia | 0 | |
| X-irradiation | + | Irreversible |

resistance. Modification of the fatty acid composition of hepatoma cell membranes has also been shown to influence cell susceptiblity to complement lysis, again implicating the membrane as an important mediator of resistance (Yoo *et al.*, 1980). These studies suggest that nucleated cells possess membrane-related mechanisms of defence against complement killing, perhaps related to membrane repair (Ohanian & Schlager, 1981).

### Stability of the functional pore of the MAC

On an inert target such as a sheep erythrocyte the functional pore formed by the MAC is a stable entity, allowing ions to flow through the pore for long periods, resulting in cell lysis (Mayer, 1977; Ramm & Mayer, 1980; Ramm *et al.*, 1983*a*). Formation of the MAC on a nucleated cell however does not necessarily result in cell lysis. MAC-induced release of $^{86}Rb$ from mouse mastocytoma cells in the absence of lysis has been demonstrated (Burakoff *et al.*, 1975), and nucleated cells bearing MACs can be 'rescued' from lysis by incubation with cyclic AMP (Kaliner & Austen, 1974; Boyle *et al.*, 1976; Lo & Boyle, 1979), raising the possibility that mechanisms of resistance and repair exist in nucleated cells. Evidence that functional pores on the membranes of nucleated cells were labile was first provided by Stephens & Henkart (1979) who showed that MAC-induced changes in membrane potential in single cultured nerve and muscle cells occurred in the absence of cell lysis and were transient. Using patch-clamping techniques the same group showed that individual MAC channels rapidly opened and closed in the cell membrane (Stephens *et al.*, 1980, Jackson *et al.*, 1981). These findings imply that nucleated cell killing, unlike that of erythrocytes, is a multi-hit phenomenon requiring many functional lesions. By comparing the numbers of MACs, formed using C6-deficient serum and limiting amounts of C6, required to lyse human erythrocytes or nucleated tumour cells it was shown that many functional MACs were required for nucleated cell lysis, suggesting the existence of mechanisms of resistance (Koski *et al.*, 1983; Ramm *et al.*, 1983*b*). Resistance mechanisms might operate either by increasing the rate of removal of ions sufficiently to overcome the MAC-induced influx, or by removing or inactivating the membrane-bound MAC thereby preventing further influx. Inhibition of ion pumps has been shown to have little effect on nucleated cell resistance to lysis, suggesting that, at least in the cell line used, the first strategy outlined above is not of major importance (Ramm *et al.*, 1984). There is now however abundant evidence for the second strategy, and this will be reviewed in the next section.

### Physical removal of MACs as a recovery strategy

Evidence that the functional lesion caused by the MAC was unstable was provided by marker molecule release studies in the human histiocytic cell line U937 (Ramm *et al.*, 1983*a*). Functional channels disappeared from the cell surface at a rate that was dependent on temperature. These results could be interpreted to indicate either that the MAC is functionally inactivated at the cell surface or that it is physically removed from the membrane. Several investigators had commented on the bizarre appearance of nucleated cells following exposure to complement, noting the presence of membrane-bound vesicles (Goldberg & Green, 1959) or of 'multiple moth-eaten protrusions' (Ohanian *et al.*, 1977) on cells that had not been lysed. Others had described the release of plasma membrane vesicles during complement attack (Richardson & Luzio, 1980; Podack & Muller-Eberhard, 1981). The possibility therefore existed that vesiculation represented a mechanism by which the cell rids itself of the potentially lytic MAC. To investigate this possibility the neutrophil was chosen as target cell because it was easily isolated and known to be resistant to killing by complement (see Fig. 1). Neutrophils bearing non-lethal amounts of the MAC were incubated at various temperatures, portions removed at intervals and the amount of the MAC remaining on the cell surface assessed by measuring subsequent binding of a radiolabelled antibody to C9 to the cell surface at 0 °C (Morgan *et al.*, 1984). MACs were rapidly removed from the cell surface in a temperature-dependent manner, the half-time of removal being about 3.5 min at 37 °C (Fig. 2). The route of removal was demonstrated to be, at least in part, via vesiculation of membrane fragments bearing many MACs (Campbell & Morgan, 1985). These results were confirmed and extended in later studies, which showed that vesiculation was the major route of MAC

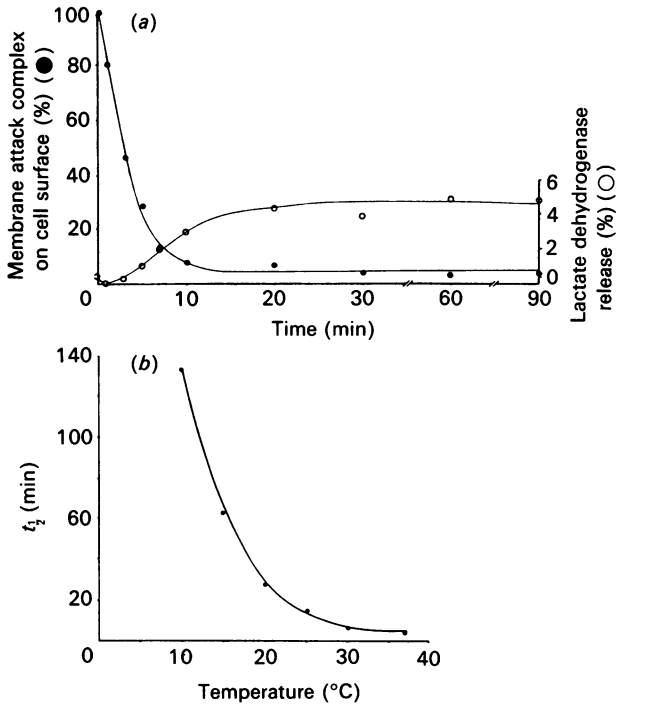


**Fig. 2. Removal of MACs from neutrophil surface membranes**

Binding of radiolabelled MC47 anti-C9 monoclonal antibody to human neutrophils bearing non-lethal amounts of the MAC was measured at intervals during incubation of these cells at 37 °C. At each time point a portion of cells was removed, placed on ice and labelled antibody added. After incubation at 0 °C cells were washed with ice-cold buffer and the amount of label associated with the cell pellet measured. (*a*) Shows the decrease in antibody binding with time of incubation at 37 °C, indicating loss of cell surface MACs in the absence of significant lysis. (*b*) Shows the temperature dependence of MAC removal, demonstrated by repeating the above experiment using different temperatures of incubation, $t_{\frac{1}{2}}$ being the time taken for binding of radiolabelled antibody to decrease to 50% of original. Modified from Campbell & Morgan (1985) with permission.

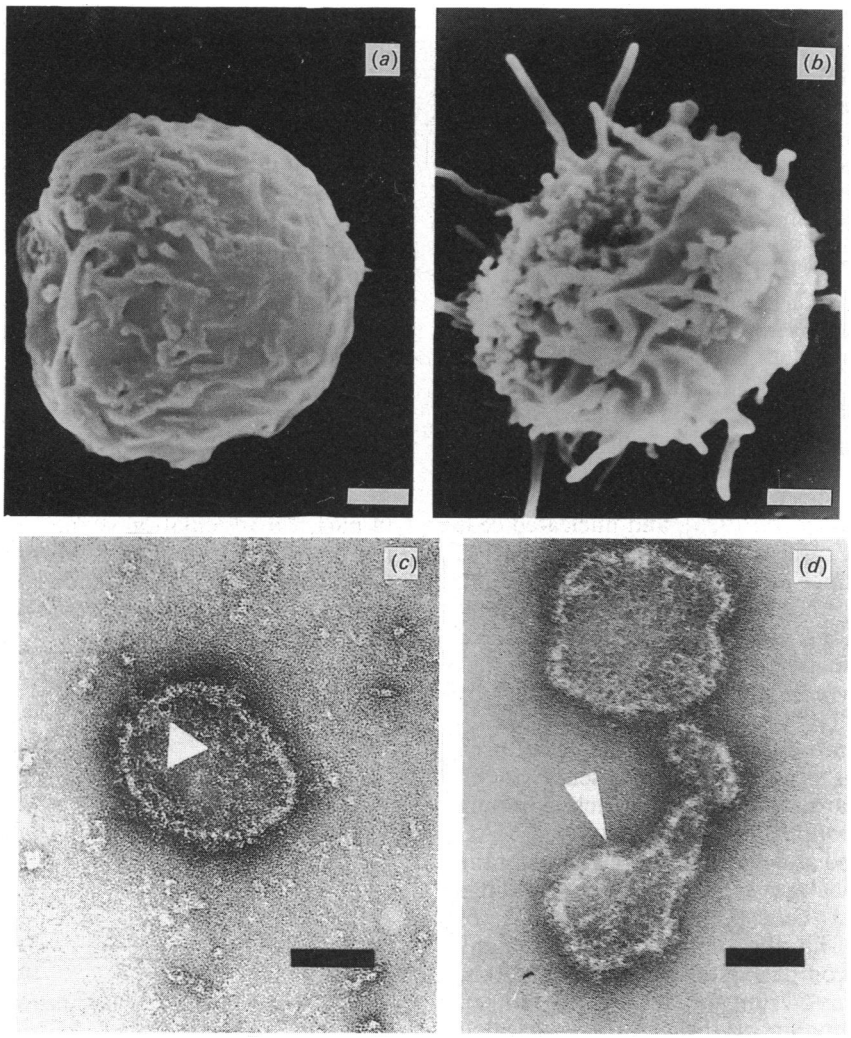


**Fig. 3. Electron microscopy of neutrophils and vesicles**

Panels (*a*) and (*b*) are scanning electron micrographs of antibody-sensitized human neutrophils prior to (*a*) or 2 min after (*b*) exposure to a non-lethal amount of human serum. Note the great increase in pseudopod formation. Panels (*c*) and (*d*) are negatively stained transmission electron micrographs of vesicles shed from neutrophils during non-lethal complement attack. Vesicles were covered with MACs; examples are arrowed in each frame. The scale bars in (*a*) and (*b*) represent 1 $\mu$m, and in (*c*) and (*d*) represent 100 nm. Modified from Morgan *et al.* (1987*b*) with permission.

removal in human neutrophils attacked with non-lethal amounts of homologous complement (Morgan *et al.*, 1987*a*). Several thousand MACs, about 65% of the total bound, were rapidly removed from the cell surface on vesicles representing just 2% of the cell surface. Fig. 3 shows in panels (*a*) and (*b*) the surface changes induced in neutrophils by non-lethal complement attack, and in panels (*c*) and (*d*) the ultrastructural appearance of the vesicles produced. Several other cell types have subsequently been shown to remove MACs by vesiculation, including human platelets (Sims & Wiedmer, 1986), rat glomerular cells (Camussi *et al.*, 1987), human synoviocytes (Morgan *et al.*, 1988*a*) and rat oligodendrocytes (Scolding *et al.*, 1989*b*). Neutrophils remove a small proportion of cell-bound MACs by endocytosis and subsequent degradation within the cell (Morgan *et al.*, 1987*a*). In some cell types, for example the Ehrlich ascites tumour cell, elimination of MACs from the cell surface occurs at a rate which is comparable with that of the neutrophil but the primary route of elimination appears to be endocytic (Carney *et al.*, 1985). The relative importance of these two routes of physical removal of MACs may therefore differ between cell types or even within a particular cell type depending on the conditions of complement attack.

## Intracellular signals for recovery processes

How then does the formation of a MAC on the surface of a cell stimulate processes within that cell which result in removal of the lesion? Perhaps the earliest detectable intracellular event caused by the MAC is a rise in intracellular free calcium ion concentration ($[Ca^{2+}]_i$) (Campbell *et al.*, 1979; Campbell & Luzio, 1981). In erythrocyte ghosts entrapping the $Ca^{2+}$-activated photoprotein obelin a MAC-dependent rise in $[Ca^{2+}]_i$ occurs within 30 s of initiating complement attack, several minutes before any detectable release of intracellular marker molecules (Morgan, 1984). In the neutrophil a similar rapid, MAC-dependent rise in $[Ca^{2+}]_i$ occurs even at non-lethal levels of complement attack

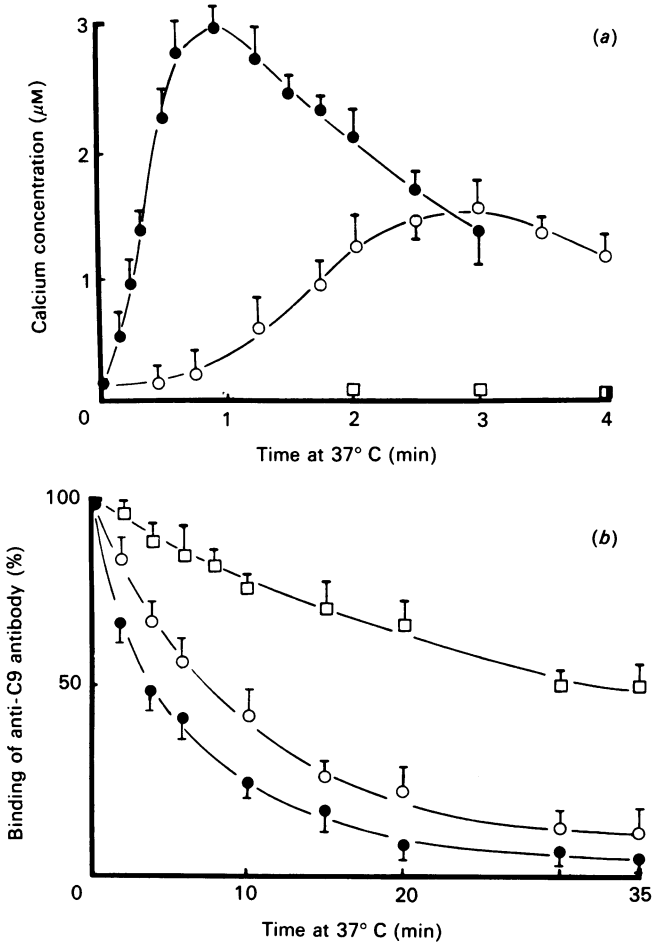


**Fig. 4. Calcium-dependence of neutrophil recovery**

(*a*) Neutrophils bearing non-lethal amounts of the MAC and entrapping the $Ca^{2+}$-activated photoprotein obelin were incubated at 37 °C in a luminometer and $[Ca^{2+}]_i$ calculated from obelin luminescence. Each point represents the average calculated value at that time in three separate experiments. Extracellular $Ca^{2+}$ 1.3 mM (●); no extracellular $Ca^{2+}$ (○); no extracellular $Ca^{2+}$, intracellular EGTA (□). (*b*) Removal of MACs from the cell surface during incubation at 37 °C was measured under each of the conditions noted above, as described in Fig. 2. In the presence of extracellular $Ca^{2+}$ (●) cell death was 5% at end point; in the absence of extracellular $Ca^{2+}$ (○) cell death was increased to 14%, and when intracellular $Ca^{2+}$ was also chelated (□) cell death was 25%. From Morgan and Campbell (1985) with permission.

(Morgan & Campbell, 1985). Again using obelin as calcium indicator, $[Ca^{2+}]_i$ rose from a basal level of 0.2 μM to a peak of about 5 μM and then returned towards the basal level (Fig. 4*a*). Using the calcium-chelating agent EGTA either outside or entrapped within the cells it was shown that most of the calcium was derived from outside the cell, presumably entering via the MAC. However, a rise in $[Ca^{2+}]_i$ occurred even in the absence of extracellular calcium, suggesting that the MAC also induced the release of calcium from intracellular stores (Fig. 4*a*). If the rise in $[Ca^{2+}]_i$ was inhibited by removing extracellular calcium after first activating complement on the cell surface, vesiculation, the primary recovery mechanism in these cells, was also inhibited, and cell lysis enhanced (Fig. 4*b*). Abolition of the rise in $[Ca^{2+}]_i$ by chelating intracellular calcium virtually eliminated vesiculation and further enhanced the lytic susceptibility of the cell (Morgan & Campbell, 1985; Morgan *et al.*, 1987*a*). Exocytic elimination of MACs by human platelets during non-lethal complement attack has also been shown to be calcium-dependent (Sims & Weidmer, 1986). Endocytic removal of MACs during recovery of Ehrlich ascites tumour cells is strongly influenced by extracellular calcium concentration and lysis of these cells by the MAC is enhanced by the chelation of $[Ca^{2+}]_i$ (Carney *et al.*, 1986; Kim *et al.*, 1987). By using Quin 2 as calcium indicator a transient increase in $[Ca^{2+}]_i$, similar to that described in the neutrophil, has been demonstrated in these cells and shown to correlate with the rate of removal of lesions. It is thus clear that in several cell types changes in $[Ca^{2+}]_i$, caused both by influx via the MAC and by release from stores, are important in the mediation of MAC removal and cell recovery from complement attack. The calcium ion is an important intracellular regulator, influencing numerous cellular metabolic pathways (Campbell, 1983). Increased $[Ca^{2+}]_i$ may act directly or via calmodulin to stimulate membrane phospholipase, cytoskeletal elements, and the production of other intracellular messengers, all of which may play a role in MAC removal.

An interesting early observation was that cyclic AMP inhibited lysis of cells by complement (Ebbsen & Arnus, 1973; Kaliner & Austen, 1974). The demonstration that cyclic AMP could 'rescue' cells bearing lytic amounts of the MAC from lysis showed that this inhibitory effect was mediated at the stage of membrane attack (Boyle *et al.*, 1976). Other cyclic nucleotides were not effective in preventing cell lysis. We have shown that the non-metabolizable adenosine agonist 2-chloroadenosine inhibits neutrophil recovery from complement membrane attack and renders the cells more susceptible to lysis (Roberts *et al.*, 1985). This effect is mediated via $A_2$-adenosine receptors which are coupled to adenylate cyclase and therefore influence intracellular cyclic AMP levels. Recently increases in intracellular cyclic AMP concentration in response to the MAC have been observed in Ehrlich ascites cells, and it has been suggested that this effect is mediated via G proteins (Carney & Shin, 1987), a group of guanine nucleotide-binding regulatory proteins which modulate adenylate cyclase activity and are probably involved in the regulation of phosphoinositide turnover (Casey & Gilman, 1988). A complex system of multiple interacting intracellular signals is therefore likely to be involved in the stimulation of recovery mechanisms in nucleated cells. The primary signals may be $[Ca^{2+}]_i$ and/or cyclic AMP, but the role of other intracellular messengers such as the inositol phosphates (Irvine, 1989) remains to be investigated.

### The extent of nucleated cell recovery

The foregoing sections demonstrate that nucleated cells can resist lysis by acute complement membrane attack, and recover by removing MACs from the cell surface. *In vivo*, complement activation on the cell surface at the site of inflammation is likely to be a chronic process. If cells are to escape lysis over a long period they will therefore need to be able to resist and recover from repeated or continuous complement membrane attack. A selective prelytic reduction of intracellular phospho-

**Table 2. Metabolic effects of non-lethal complement attack**

Cell viability, energy content and functional parameters were measured in antibody-sensitized neutrophils (pre-attack), in PAC1-9 cells immediately following protection by removal of cell bound MACs (protected), and in protected cells which had subsequently been allowed to recover by incubation in nutrient medium (recovered). All results are the means of three separate experiments, with S.D. in parentheses. From Morgan (1988) with permission.

| Parameter measured | Pre-attack | Protected | Recovered |
|---|---|---|---|
| Trypan Blue exclusion (% positive) | 3 (1) | 5 (2) | 6 (1) |
| Lactate dehydrogenase activity (units/$10^9$ cells) | 13 (0.5) | 12 (0.4) | 12 (0.7) |
| ATP content | 0.4 (0.1) | 0.09 (0.04 | 0.4 (0.1) |
| Reactive oxygen metabolite production (% of pre-attack) | 100 | 22 (5) | 89 (8) |
| Chemotactic index | 58.2 (3.8) | 16.4 (2.9) | 51.4 (3.7) |
| Phagocytosis (c.p.m./cell) | 42 (2) | 8 (0.5) | 47 (1.5) |

creatine and ATP, both important energy sources, has been demonstrated in tumour cells attacked by antibody and complement and a role of energy depletion in eventual cell lysis has been suggested (Tirosh *et al.*, 1984). In neutrophils, ATP depletion occurs during non-lethal complement membrane attack and is accompanied by an inhibition of energy-requiring processes such as chemotaxis, reactive oxygen metabolite production and phagocytosis, and by cell swelling (Morgan, 1988). However, all these effects are rapidly reversed when the cells are placed in a nutrient-containing medium, and recovered cells are just as resistant to further complement attack as are previously unattacked cells, demonstrating that complete biochemical and functional cell recovery occurs (Table 2). In oligodendrocoytes, complete recovery from a single episode of complement membrane attack has similarly been shown, cells continuing to express sequentially developing cell surface markers at the same time as unattacked cells (Scolding *et al.*, 1989*a*). In this case, however a cumulative effect of repeated attack was observed, the percentage of surviving cells being lysed increasing with each episode of attack despite apparently complete restoration of cellular energy stores. The extent of recovery, as judged by the metabolic status, continued growth or development and ability to resist repeated or continuous low-level attack by the MAC, is therefore likely to vary in different cell types. The capacity to recover fully will allow a cell to continue to function at sites of complement activation, a factor of particular relevance to neutrophils which congregate at inflammatory sites.

### MAC-inhibiting proteins and nucleated cell recovery

Nucleated cells, like erythrocytes, are far more susceptible to lysis by heterologous complement than by homologous. This phenomenon of 'homologous restriction' was first noted over 70 years ago (Muir, 1911). The identification of species-specific cell-surface inhibitors of the activation pathways of complement provided a mechanistic explanation for this phenomenon (Hoffman, 1969*a*,*b*; Nicholson-Weller *et al.*, 1982), but did not explain homologous restriction occurring using the reactive lysis system, a strategy which by-passes the activation pathways (Lachmann *et al.*, 1973; Hansch *et al.*, 1981). Recently, several groups have reported the isolation from erythrocyte membranes of proteins with MAC-inhibiting activity which are to some degree species-specific (Schonermark *et al.*, 1986; Zalman *et al.*, 1986; Watts *et al.* 1987; Sugita *et al.*, 1988). These proteins bind to terminal complement components and inhibit cell lysis by the MAC. It appears that at least two distinct proteins exist on erythrocyte membranes, one with a molecular mass of approx. 60 kDa (homologous restriction factor, C8-binding protein, MAC-inhibiting protein), and another protein of molecular mass 18 kDa (p-18). The larger protein has also been identified on and/or isolated from platelets, monocytes and lymphocytes (Blaas *et al.*, 1988; Martin *et al.*, 1988), and is likely to be present on many other cell types. A fluid-phase form of the protein has also recently been described (Watts *et al.*, 1989). As these proteins bind to terminal complement components and inhibit lysis it is probable that they become physically incorporated into the MAC during its assembly. Preliminary evidence of incorporation of inhibitory protein into the MAC has been obtained by density gradient centrifugation of MACs formed on sheep cells in the presence of the radiolabelled protein (Schonermark *et al.*, 1988). Using immunoelectron microscopy we have recently localized endogenous MAC-inhibiting protein in the MAC on human cells following attack with homologous complement (J. R. Dankert, M. J. Watts & B. P. Morgan, unpublished work). Whether the inhibitory protein acts by preventing functional channel expression or by enhancing MAC elimination from the cell surface is uncertain; however, vesicles shed from neutrophils during recovery are covered with ring-like MACs yet do not release entrapped marker molecules (B. P. Morgan, unpublished work), suggesting that the lesions are plugged prior to removal on vesicles.

A schematic diagram of the important mechanisms and routes of nucleated cell recovery from non-lethal complement membrane attack is shown in Fig. 5.

## NON-LETHAL EFFECTS OF THE MAC

The data reviewed in the preceding sections demonstrate that nucleated cells, because of the existence of efficient mechanisms of resistance and repair, are relatively difficult to kill with the MAC, particularly when

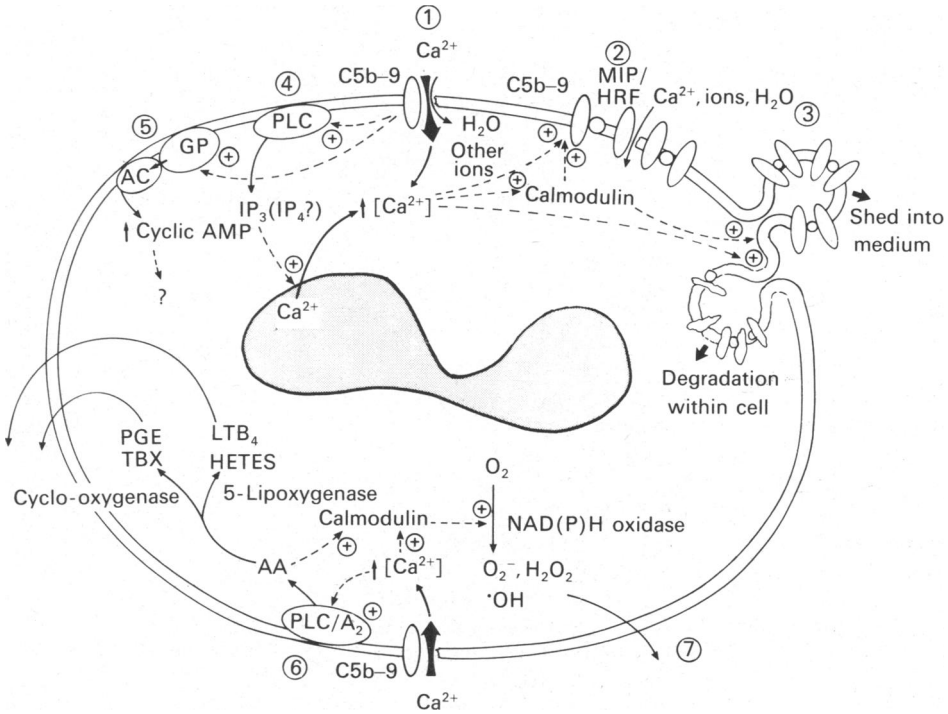


**Fig. 5. Mechanisms of cell recovery and activation by the MAC**

In this schematic representation of a nucleated cell during non-lethal complement membrane attack recovery mechanisms are illustrated in the upper half of the cell and non-lethal effects in the lower. MAC formation on the cell surface causes influx of $Ca^{2+}$ and elevation of $[Ca^{2+}]_i$ ①, which directly or via calmodulin stimulates aggregation of MACs ② and eventual removal by endocytosis and exocytosis ③. MAC-inhibitory proteins (MIP, HRF) may act by inhibiting leakage through the MAC or by enhancing removal. The MAC also stimulates phospholipase C (PLC) resulting in inositol phosphate ($IP_3$, $IP_4$) production and release of $Ca^{2+}$ from intracellular stores ④ and, directly or via $Ca^{2+}$, activate G-protein systems resulting in adenylate cyclase (AC) activation and cyclic AMP production ⑤. Increased $[Ca^{2+}]_i$ by activation of phospholipases C and $A_2$ and of 5-lipoxygenase causes increased production of arachidonic acid (AA) metabolites ⑥ and, via calmodulin, stimulates reactive oxygen metabolite production ⑦.

homologous complement is used. What then are the effects, if any, of non-lethal amounts of the MAC on nucleated cells? Some effects have already been described. The MAC causes depletion of cellular energy stores prior to or in the absence of lysis (Tirosh *et al.*, 1984; Morgan, 1988; Scolding *et al.*, 1989), and also causes reversible cell swelling (Morgan, 1988). Both of these effects may be considered as inevitable consequences of ion and water leak through the functional pore of the MAC prior to its elimination. Several more specific stimulatory effects of the MAC have been reported. These effects will be described in this section and their possible pathogenic significance discussed.

### Pro-inflammatory effects

One of the first specific non-lethal effects of the MAC to be described was the stimulation of neutrophil reactive oxygen metabolite production (Hallett *et al.*, 1980, 1981). In these early experiments release of reactive oxygen metabolites, measured using luminol-enhanced chemiluminescence, was shown to occur prior to any detectable cell lysis and was dependent upon the presence of all MAC component proteins and extracellular $Ca^{2+}$ (Campbell *et al.*, 1981; Campbell & Hallett, 1983). This effect was subsequently shown to occur even at doses of the MAC which caused no lysis of neutrophils, demonstrating that it was a true non-lethal effect of the MAC (Morgan, 1984; Morgan & Campbell, 1985). Stimulation of reactive oxygen metabolite production by non-lethal amounts of the MAC has subsequently been demonstrated in a variety of other nucleated cell types (summarized in Table 3). These metabolites are highly toxic to cells, causing a broad range of deleterious effects, thoroughly reviewed in this journal by Halliwell & Gutteridge (1984) and by Slater (1984), which may under differing circumstances be reversible, causing temporary cell dysfunction, or irreversible, causing eventual cell death (Hinshaw *et al.*, 1987).

Non-lethal amounts of the MAC have also been shown to stimulate the synthesis and release of metabolites of arachidonic acid, including prostaglandins, thromboxanes, leukotrienes and hydroxyeicosatetraenoic acids (HETEs) from a variety of nucleated cells *in vitro* and *in vivo*. These substances are intimately involved in the mediation of pain and inflammation *in vivo* (Davies *et al.* 1984; Salmon & Higgs, 1987). Table 3 lists the cell types in which MAC-induced stimulation of production of these highly reactive inflammatory mediators has been demonstrated. These effects occur independently of the well-known stimulation of arachidonate metabolism by the complement cleavage products C3a, C3b and C5a (Hartung *et al.*, 1983; Stimler *et al.*, 1982), and are dependent on MAC formation. Some groups report that the C5–8 complex is a sufficient stimulus, addition of C9

**Table 3. Non-lethal effects of the MAC**

Abbreviations: ROM, reactive oxygen metabolites; $LTB_4$, leukotriene $B_4$; $TXB_2$, thromboxane $B_2$; $PGE_2$, prostaglandin $E_2$; PL, phospholipase; IL-1, interleukin 1; NI, not investigated.

| Cell | Effects | $Ca^{2+}$ dependence | Reference |
|---|---|---|---|
| Neutrophil (rat) | ROM production | ✓ | Morgan *et al.* (1984) |
| | $LTB_4$ production | ✓ | Imagawa *et al.* (1987) |
| Neutrophil (human) | ROM production | ✓ | Morgan & Campbell (1985) |
| | $LTB_4$ production | ✓ | Seeger *et al.* (1986) |
| | Vesiculation | ✓ | Campbell & Morgan (1985) |
| Platelet (human) | Prothrombinase activation | ✓ | Wiedmer *et al.* (1986) |
| | Vesiculation | ✓ | Sims & Wiedmer (1986) |
| | $TXB_2$ production | −* | Betz *et al.* (1987) |
| Monocyte/macrophage (human) | $PGE_2 + TXB_2$ production | ✓ | Hansch *et al.* (1984) |
| | ROM production | ✓ | Hansch *et al.* (1987) |
| Glomerular epithelial cell (rat) | $PGE_2 + TXB_2$ production | NI | Hansch *et al.* (1988) |
| | Vesiculation | NI | Camussi *et al.* (1987) |
| | PL activation + $IP_3$ production | ✓ | Cybulsky *et al.* (1988) |
| Glomerular mesangial cell (rat) | $PGE_2$ + IL-1 production | NI | Lovett *et al.* (1987) |
| | ROM production | NI | Adler *et al.* (1986) |
| Oligodendrocyte (rat) | $LTB_4$ production | NI | Shirazi *et al.* (1987) |
| Synoviocyte (human) | ROM production | ✓ | Morgan *et al.* (1988*a*) |
| | $PGE_2$ production | NI | Daniels *et al.* (1989) |
| Tumour cell lines | $LTB_4$ production | ✓ | Imagawa *et al.* (1983) |
| | Vesiculation | ✓ | Kim *et al.* (1987) |

* Extracellular $Ca^{2+}$ not required, but release of $Ca^{2+}$ from intracellular stores does occur.

causing little or no enhancement of metabolite release (Seeger *et al.*, 1986), whereas others find a requirement for the complete MAC (Imagawa *et al.*, 1987). The nature of the metabolite produced varies from cell to cell (Table 3). The likely explanations for and physiological significance of these differences will be discussed later.

A third group of inflammatory mediators, the cytokines, consist of a family of about 50 molecules and includes interleukin 1, produced by a variety of cell types, and tumour necrosis factor, produced mainly by lymphocytes and macrophages (Billingham, 1987). The effects of non-lethal complement membrane attack on the synthesis of these mediators has been investigated by several groups and increased interleukin 1 secretion in response to the MAC has been demonstrated in human monocytes (Hansch *et al.*, 1987) and in rat glomerular mesangial cells (Lovett *et al.*, 1987).

## Mechanisms of induction of proinflammatory effects

Non-lethal amounts of the MAC can therefore, at least in some nucleated cell types, stimulate the production of inflammatory mediators, including reactive oxygen metabolites, metabolites of arachidonic acid and cytokines. By what pathways does the MAC stimulate these processes? As described above, one of the earliest detectable intracellular events on formation of the MAC on a cell is a rise in $[Ca^{2+}]_i$ (Campbell *et al.*, 1979), and this rise has been implicated in cell recovery processes (Morgan & Campbell, 1985). These findings make $[Ca^{2+}]_i$ a prime candidate as a mediator of other non-lethal effects of the MAC. MAC-induced reactive oxygen metabolite production by neutrophils, macrophages, monocytes and synoviocytes is dependent on the presence of extracellular $Ca^{2+}$ (see Table 3 for references), suggesting a role for this ion in the mediation of their production. Further, the calcium ionophore A23187 mimics the stimulatory effects of non-lethal amounts of the MAC in neutrophils (Hallett *et al.*, 1981), implying that influx of $Ca^{2+}$ via the functional pore of the MAC is, at least in this case, the initiating factor. The increase in $[Ca^{2+}]_i$ could act via calmodulin to cause stimulation of NADPH oxidase, a key enzyme in the neutrophil respiratory burst pathway, resulting in the observed release of metabolites (Klebanoff & Clark, 1978; Campbell, 1984). Although firm evidence that MAC-induced reactive oxygen metabolite production in all cell types is mediated by $[Ca^{2+}]_i$ is not available, calcium-independent stimulation by the MAC has not been demonstrated in any cell in which it has been looked for (see Table 3).

The situation with regard to arachidonic acid metabolite production is somewhat less clear. The biosynthetic pathways involved are complex; the initial and rate-limiting step is the phospholipase-catalysed release of arachidonic acid from membrane lipid. Thereafter, arachidonic acid may be metabolized along one of two pathways, the cyclo-oxygenase pathway, widely distributed in mammalian cells and producing prostaglandins or thromboxanes, or the 5-lipoxygenase pathway, restricted to neutrophils, eosonophils, macrophages, monocytes and mast cells, and producing leukotrienes and HETEs (Salmon & Higgs, 1987). A role for calcium in control of these pathways is suggested by the observations that stimulation of the arachidonic acid cascade in macrophages by many stimuli is dependent on the presence of extracellular calcium and is inhibited by calcium antagonists like quinine (Aderem *et al.*, 1986), and that

the calcium ionophore A23187 stimulates arachidonic acid release in neutrophils (Meade *et al.*, 1986). Calcium may be involved at several stages.

Firstly, it may non-selectively increase the production of metabolites by increasing the availability of arachidonic acid as substrate, either by activating phospholipase or by inhibiting lysophosphatide acyltransferase which re-forms phospholipid from lysophospholipid and fatty acid (Irvine, 1982). The calcium ionophore A23187 stimulates via activation of phospholipase $A_2$ (Meade *et al.*, 1986) and MAC-induced phospholipase activation has recently been demonstrated in glomerular epithelial cells and shown to be calcium-dependent (Cybulsky *et al.*, 1988*b*). Removal of extracellular $Ca^{2+}$ abolishes MAC-induced secretion of arachidonic acid metabolites in neutrophils and monocytes, suggesting a similar mechanism (Imagawa *et al.*, 1987; Hansch *et al.*, 1987). In platelets, where the MAC is thought to act by inhibiting the arachidonic-acid-removing enzyme lysolecithin acyltransferase (Hansch *et al.*, 1987), influx of $Ca^{2+}$ is not required for induction of release of metabolites (Betz *et al.*, 1987), implying that $Ca^{2+}$ is not the primary signal. However, increased $[Ca^{2+}]_i$ as a result of release from intracellular stores does occur and may therefore still be important in the initiation of metabolite secretion.

Secondly, calcium may selectively activate the calcium-dependent 5-lipoxygenase pathway in those cells that possess it, resulting in selective enhancement of synthesis of leukotrienes and HETEs (Salmon & Higgs, 1987). Because of the diverse effects caused by these highly active mediators, subtle alterations in the products of activation may be at least as important as the non-specific stimulation of the pathway.

Synergistic interaction between the pro-inflammatory products of non-lethal complement membrane attack will occur *in vivo*, further enhancing the inflammatory response. A mediator may even enhance the production of other mediators. Arachidonic acid metabolites for example directly stimulate reactive oxygen metabolite production in neutrophils by activating NAD(P)H oxidase (Curnutte *et al.*, 1984). The mechanisms and pathways involved in inflammatory mediator production by nucleated cells in response to non-lethal amounts of the MAC are represented in Fig. 5.

## THE ROLE OF THE MAC IN DISEASE

In this final section an attempt will be made to place the preceeding observations, made on cells *in vitro*, into the context of their significance to disease pathology. Evidence for MAC involvement in a variety of disease processes will be presented and the mechanisms by which it may contribute to the pathology of disease explored.

### Involvement of the MAC in disease

The MAC may be implicated in a disease process by the demonstration of: increased consumption of terminal complement components or production of terminal complement complexes (SC5b–9 or MAC) in biological fluids; localization of the MAC in diseased tissue; or abrogation of the disease by inhibition of MAC-producing capacity (Morgan, 1989). The availability of antibodies specific to neoantigens of terminal complement complexes (TCCs) has made their measurement in biological fluids and localization in diseased tissue a relatively simple process and has stimulated an explosion of interest in TCCs, reflected in a large number of publications, and an increasingly long list of diseases in which TCCs are implicated (Table 4).

Anti-neoantigen antibodies do not distinguish between the two types of TCC, the MAC and the inactive SC5b–9 complex. Attempts to measure specifically each complex in tissues or in the fluid-phase have been made (Mollnes & Harboe, 1987; Morgan *et al.*, 1988*b*). However, in most cases activation of the terminal complement pathway will result in the formation of both tissue-bound (and fluid-phase?) MACs and fluid-phase (and tissue-bound?) SC5b–9 complexes, rendering the distinction irrelevant and implicating the cytopathic MAC in all diseases where serum concentrations of TCCs are elevated or TCCs are localized in tissues.

In these diseases the MAC may contribute to tissue injury either primarily, by disrupting membranes and causing cell death, or secondarily by stimulating cells to produce toxic pro-inflammatory mediators. From the preceeding discussions it should be clear that killing of nucleated target cells by homologous MACs *in vivo* is not likely to be of major importance in many diseases, resistance and recovery mechanisms allowing cells to survive unless overwhelmed. The absence of severe tissue necrosis in most diseases in which the MAC is implicated supports this contention. Non-lethal pro-inflammatory effects are however likely to occur *in vivo* and may be of importance in disease pathogenesis. The following sections will examine the evidence for MAC involvement in a few diseases chosen as examples to illustrate principles which may be applicable to many diseases.

### Neurological and neuromuscular diseases

The first disease in which the MAC was implicated was myasthenia gravis, an autoimmune disease characterized by the loss of acetylcholine receptors from the motor end plate, resulting in extreme muscle fatiguability. Anti-C9 antibodies were used to localize the MAC at the end plate, and a role of the MAC in acetylcholine receptor loss proposed (Sahashi *et al.*, 1980). Recently, these findings have been confirmed using antibodies to neoantigens of the MAC (Engel & Arahata, 1988). A requirement for terminal complement components, specifically C6, has been demonstrated in a model of myasthenia gravis, experimental myasthenia induced in rats by inoculation with antibodies to the acetylcholine receptor (Biesecker, 1987), thus demonstrating that, at least in this model, MAC formation is essential for disease expression. It has been suggested that the MAC causes 'focal lysis' of the junctional membrane resulting in loss of membrane and acetylcholine receptors (Biesecker, 1983). A more likely explanation in the light of our present knowledge is that membrane and receptors are lost together with the MAC as a result of recovery processes.

Multiple sclerosis is a common neurological disease characterized by widespread demyelination within the central nervous system. The pathogenesis of the disease is obscure, though many factors have at various times been implicated. The first evidence suggestive of a role of the MAC came from the demonstration that the concentration of the terminal complement component C9 was reduced in cerebrospinal fluid from patients with this disease, implying its utilization in MAC formation (Morgan *et al.*, 1984*a*). Subsequently, elevated levels of

**Table 4. Diseases in which the MAC is implicated**

The MAC has been implicated in numerous diseases by the demonstration of elevated levels of TCCs in biological fluids (column 1), by localization of the MAC in diseased tissue (column 2) and/or by demonstration of a requirement for complement for disease expression in animal models (column 3).

| | TCCs | MAC localization | Complement-dependence | Reference |
|---|---|---|---|---|
| Neurological/neuromuscular | | | | |
| Myasthenia gravis | | ✓ | ✓ | Sahashi *et al.* (1980), Biesecker (1987) |
| Multiple sclerosis | ✓ | ✓ | ✓ | Sanders *et al.* (1986*b*), Compston *et al.* (1989), Linington *et al.* (1989) |
| Cerebral lupus | ✓ | | | Sanders *et al.* (1987) |
| Guillain-Barre syndrome | ✓ | | | Koski *et al.* (1987) |
| Renal | | | | |
| Lupus nephritis | ✓ | ✓ | | Biesecker, (1983), Mollnes & Harboe (1987), Horigome *et al.* (1987) |
| Membranous glomerulonephritis | ✓ | ✓ | ✓ | Cosyns *et al.* (1986), de Heer *et al.* (1985) |
| Nephrotic syndrome | | ✓ | | Rus *et al.* (1986) |
| IgA nephropathy | | ✓ | | Rauterberg *et al.* (1987) |
| Dermatological | | | | |
| Pemphigoid | | ✓ | | Dahl *et al.* (1984) |
| Dermatitis herpetiformis | | ✓ | | Dahl *et al.* (1985) |
| Dermatomyositis | | ✓ | | Kissel *et al.* (1986) |
| Rheumatological | | | | |
| Rheumatoid arthritis | ✓ | ✓ | | Morgan *et al.* (1988*b*), Mollnes & Paus (1986), Sanders *et al.* (1986*a*) |
| Behcet's disease | ✓ | | | Morgan *et al.* (unpublished work) |
| Others | | | | |
| Atheroma | | ✓ | | Niculescu *et al.* (1987) |
| Myocardial infarction | | ✓ | | Schafer *et al.* (1986) |
| Autoimmune thyroid disease | ✓ | ✓ | | Weetman *et al.* (1989) |

TCCs have been reported in the cerebrospinal fluid (Sanders *et al.*, 1986*b*; Mollnes *et al.*, 1987), and the MAC localized in brain tissue (Compston *et al.*, 1989) in multiple sclerosis. We have recently shown that cerebrospinal fluid from patients with multiple sclerosis contains not only fluid-phase SC5b–9 complexes, but also MACs on oligodendrocyte-derived membrane vesicles, providing evidence *in vivo* for the occurrence of non-lethal complement attack and cell recovery (Scolding *et al.*, 1989*b*). Experimental allergic encephalomyelitis in rats is utilized as a model for multiple sclerosis despite the fact that it is a purely inflammatory disease. A demyelinating disease more closely resembling multiple sclerosis can be induced in animals with allergic encephalomyelitis by inoculation with a specific anti-myelin antibody. In both forms of the disease, decomplementation of rats prior to the onset of symptoms markedly ameliorates the clinical course and prevents demyelination, suggesting a role of complement in this disease (Linington *et al.*, 1989).

Non-lethal effects of the MAC already demonstrated at the cellular level *in vitro* (Shirazi *et al.*, 1987; Scolding *et al.*, 1989*a*) may therefore be, at least in part, responsible for the relapsing and remitting course of the clinical disease. Fig. 6 illustrates in (*a*) the elevated concentration of TCCs in the cerebrospinal fluid and in (*b*) the presence of MACs in brain tissue of patients with multiple sclerosis.

**Rheumatological diseases**

The MAC has been implicated in a number of rheumatological diseases (Table 4). In rheumatoid arthritis elevated levels of TCCs have been found in synovial fluid and in plasma (Mollnes *et al.*, 1986; Mollnes & Paus, 1986; Morgan *et al.*, 1988*b*) and the MAC has been localized in rheumatoid synovium (Sanders *et al.*, 1986*a*). In rheumatoid joints release of inflammatory mediators including arachidonic acid metabolites and cytokines into synovial fluid has been demonstrated and a role of these mediators in the causation or propagation of inflammation suggested (Hopkins *et al.*, 1988). We have shown that non-lethal amounts of the MAC stimulate human synoviocytes *in vitro* to secrete prostaglandins and reactive oxygen metabolites (Morgan *et al.*, 1988*a*;

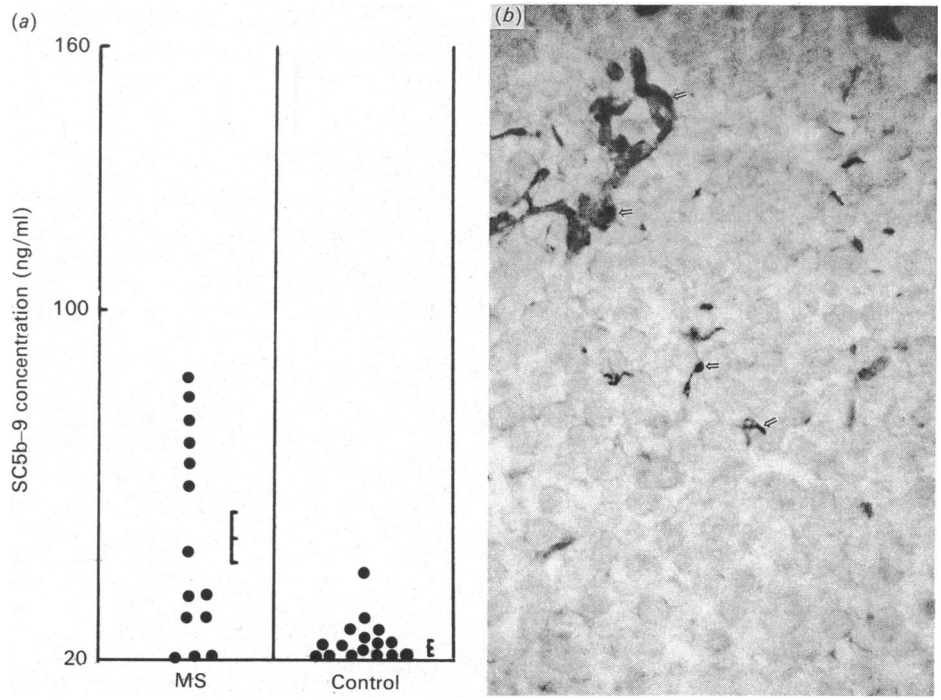


**Fig. 6. Cerebrospinal fluid and tissue TCCs in multiple sclerosis**

Panel (*a*) compares the concentrations of TCCs in the cerebrospinal fluid of 14 patients with multiple sclerosis (MS) with those in 16 control individuals. The mean concentration in patients (28.6 ng/ml) was significantly elevated when compared with controls (2.9 ng/ml). Panel (*b*) shows a section of a plaque from the brain of a patient with multiple sclerosis stained with anti-neoantigen antibody to identify TCCs. Granular staining is present in capillaries (arrowed). Control tissue showed no specific staining for TCCs.

Daniels *et al.*, 1989). These results provide a link between the observations *in vivo* of MAC localization and inflammatory mediator production in rheumatoid synovium and firmly implicate the MAC in disease pathogenesis.

## CONCLUDING REMARKS

In this review I have attempted to introduce the concept that the MAC can act as a cell-stimulatory agent rather than being merely a cell-killing agent. The MAC can, of course, under appropriate conditions cause lysis of cells and lysis of cells by the MAC may be important in the pathogenesis of some diseases. However, the phenomena of homologous restriction and of nucleated cell resistance to lysis by the MAC serve to minimize killing of homologous cells *in vitro* and *in vivo*. Non-lethal amounts of the MAC will nevertheless be deposited on the surfaces of cells at sites of complement activation and will stimulate them, resulting in release of pro-inflammatory factors, potentiating inflammation.

As shown in Table 4, the MAC has been implicated in a large and diverse group of diseases. To suggest that the MAC is the causative agent in all these diseases would be naive. However, even as a secondary factor, enhancing and prolonging tissue damage and inflammation initiated by other effectors, the MAC may play a significant part in disease pathogenesis and may, as is the case in several of the animal models of disease described above, be essential for the disease to be expressed clinically. Therapeutic strategies which inhibit or prevent MAC formation at the site of complement activation might therefore be of benefit in many diseases. Naturally occuring inhibitors of the MAC exist in plasma and on membranes and one possible strategy would be to develop drugs which mimic their effects or enhance their expression *in vivo*.

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