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The Role of Complement in Neuronal Cell Turnover in the Central Nervous System

PhD Thesis

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This thesis is dedicated to my family, for whom my research was never just a day job.

Contents

Abstract

Complement (C) activation and neuronal apoptosis occur in areas of active pathology in both neuroinflammatory and neurodegenerative disease. Little is currently known about how these two processes interact, and whether this causes further damage or promotes resolution and repair. The hypothesis of this thesis was that C modulates neuronal cell turnover in the central nervous system, with particular emphasis on its role in modulating apoptosis and handling of apoptotic cell debris. I have shown that apoptotic cells activated C more readily than controls, a change mediated via cleavage of CD46 from the cell membrane by matrix metalloproteases; this resulted in increased phagocytosis and, in the early stages, increased C-mediated lysis, followed at later time points by decreased C-mediated killing. Cells surviving the apoptotic insult were also more resistant to C-mediated killing. CD59 was shed from apoptotic cells on blebs and as a soluble form, but cell surface levels were maintained late into apoptosis. The second part of the thesis examined the role of the C membrane attack complex (MAC) in modulating neuronal cell death. A reactive lysis system was developed, and neuronal cells were shown to possess recovery mechanisms on sublytic MAC attack. Neuronal cells subject to sublytic C displayed induced protection to lytic C, and this was shown to be MAC-independent. No effect of sublytic C was seen on neuronal apoptosis. The last part of the thesis used a kainic acid model of excitotoxicity in rats, and showed that the presence of the MAC did not have a significant role in modulating neuronal apoptotic death, but may have a role in sensitisation to seizures. Apoptotic neurons bound more C activation products and also stained brightly for CD59. These results demonstrate that C plays an important role in determining apoptotic cell fate, and when present at sublytic levels may promote neuronal survival and facilitate repair.

Publication List

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List of abbreviations:

AD	Alzheimer's disease
ADAM	a disintegrin and metalloprotease
ADEAE	antibody dependent experimental autoimmune encephalomyelitis
AP-1	activator protein 1
APAF-1	apoptotic peptidase activating factor 1
BBB	Blood brain barrier
C	complement
C#	complement component number #
C8d	C8 depleted human serum
CD#	cluster of differentiation number #
CFD	complement fixation diluent
CNS	central nervous system
CPT	camptothecin
CReg	complement regulator
CRP	C reactive protein
CSF	cerebrospinal fluid
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
E	erythrocyte
EAE	experimental autoimmune encephalomyelitis
EDTA	ethylenediaminetetraacetic acid
EGTA	Ethylene glycol-bis(2-aminoethylether)-*N,N,N',N'*-tetraacetic acid
FACS	fluorescence activated cell sorter
FCS	foetal calf serum
FFP	fresh frozen plasma
FITC	fluorescein isothiocyanate
GBS	Guillain-Barre syndrome
HI-NHS	heat inactivated normal human serum
IL-#	interleukin number #
IP	intraperitoneal
JNK	c-Jun N-terminal kinase

KA	kainic acid
LDH	lactate dehydrogenase
mAb	monoclonal antibody
MAC	membrane attack complex
MAPK	mitogen activated protein kinase
MASP	MBL associated serine protease
MBL	mannose binding lectin
MFI	mean fluorescence intensity
MMP	matrix metalloprotease
MS	multiple sclerosis
NADH	nicotinamide adenine dinucleotide (reduced)
NFκB	nuclear factor kappa B
NFT	neurofibrillary tangle
NHS	normal human serum
NMDA	N-methyl-D-aspartic acid
PBS	phosphate buffered saline
PEG	polyethylene glycol
PI	propidium iodide
PMSF	Phenylmethanesulfonyl fluoride
PNS	peripheral nervous system
RNA	ribonucleic acid
RPE	recombinant phycoerythrin
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SLE	systemic lupus erythematosus
s/n	supernatant
ssDNA	single stranded deoxyribonucleic acid
TCA	trichloroacetic acid
TIMP	tissue inhibitor of metalloproteases
TNF-α	tumour necrosis factor alpha
TUNEL	Tdt-mediated dUDP nick end labelling
UV	ultraviolet
VBS	veronal buffered saline

Chapter 1: Introduction

1. The Clinical Scenario

1.1. Neuroinflammation and neurodegeneration

At the turn of the 21st century, diseases such as multiple sclerosis (MS) and Alzheimer's disease (AD) remain a significant cause of neurological disability and handicap in the Western world. Numerous pathogenic mechanisms are at play in such disorders, including inflammation, metabolic stress and abnormal protein processing, but they both converge on a common end-point: neuronal death. It is becoming increasingly recognised that neuronal loss is strongly correlated with functional deficit and disability in these diseases (Davie, Barker et al. 1995; Bjartmar, Wujek et al. 2003), and is thus an important focus for research and efforts to develop novel therapeutics.

1.2. Complement (C) activation and neuronal apoptosis in the brain

When neurons die in the brain, they can do so either traumatically by necrosis or in an orderly fashion by apoptosis. In animal models of MS it has been shown that neuronal apoptosis is a feature of pathology, and that functional deficit follows from neuronal loss by this mechanism (Meyer, Weissert et al. 2001; Hobom, Storch et al. 2004). However, the C system is also activated in the brains of patients with MS: C activation products are detectable in inflammatory lesions in the brain, and also

in the patient's cerebrospinal fluid (CSF) (Compston, Morgan et al. 1989; Scolding, Morgan et al. 1989). As part of its myriad functions in the body, C has the capability to kill cells via the insertion of the membrane attack complex (MAC) and cause necrosis. When disease processes were studied in animal models of MS, such as experimental autoimmune encephalomyelitis (EAE) and antibody-dependent EAE (ADEAE), C inhibition was shown to be neuroprotective and axonal loss and demyelination were shown to be dependent on the formation of the MAC (Piddlesden, Storch et al. 1994; Mead, Singhrao et al. 2002). Thus both forms of cell death occur under pathological conditions in the CNS.

1.3. Rationale for the study

Although both necrotic and apoptotic neuronal death occur in neuroinflammatory disease, they do not operate in isolation. Both have been demonstrated to be involved in pathology, and both occur in active inflammatory lesions (Compston, Morgan et al. 1989; Dowling, Husar et al. 1997). It is therefore important to understand more fully the interaction between apoptosis and C activation in neuronal cells, and the consequences for neurons when C is activated but not lethal (so-called sublytic attack). Only by doing so will we discover how the balance between these processes may affect the progression of disease and how best to tackle the problem therapeutically.

The remainder of this introductory chapter will discuss both the process of apoptosis and the functions of the C system, and will review what is currently known about their interaction and their role in CNS pathology.

2. Apoptosis

2.1. Spectrum of cell death

Cell death can be broadly split into two groups – "accidental" cell death, such as necrosis, or "programmed" cell death, which includes apoptosis. These different types of cell death have characteristic morphological and biochemical features. Necrosis is usually associated with a severe insult to the cell, resulting in a catastrophic metabolic crisis such that energy-independent death mechanisms are initiated. The process of necrosis is extremely quick compared to other death mechanisms, taking a matter of minutes. Typically, cells become enlarged and organelles become swollen. The nucleus condenses, and the cell loses membrane integrity, spilling cellular contents into the surrounding tissue. This explosive type of death is highly pro-inflammatory, activating surrounding leukocytes and innate immune mediators. Thus necrosis can be viewed as a destructive, inflammatory event, and is involved in numerous pathological processes.

Apoptosis, in contrast, is classically a non-inflammatory form of cell death, and occurs under numerous physiological conditions such as embryonic development, tissue remodelling, and in the selection of T cells in the thymus. Apoptosis is a form of programmed cell death, and as such it requires energy. Typically, apoptosis takes a matter of hours, and as the cell dies it packages up its contents into apoptotic bodies, which retain membrane integrity until they are removed by phagocytes. Apoptotic cells are usually rapidly cleared by neighbouring cells, but if this does not occur, for example if the load of apoptotic cells is too great and overwhelms the capacity of phagocytes to clear them, then they enter a late phase of apoptosis and lose membrane integrity. Thus failure to clear debris can turn a non-inflammatory mode of cell death into a pro-inflammatory one.

2.2. Characteristics of apoptosis

Cells undergoing apoptosis were first described by Kerr et al (Kerr, Wyllie et al. 1972) as displaying a number of characteristic morphological features, which include cellular shrinkage, chromatin condensation and cell surface blebbing. These changes are orchestrated by the activation of a family of proteases known as caspases, which cleave various substrates in the cell (Earnshaw, Martins et al. 1999). Morphological changes are mediated by the cleavage of the cytoskeletal protein gelsolin (Kothakota, Azuma et al. 1997), and nuclear structure alters with the cleavage of lamin (Rao, Perez et al. 1996). Apoptotic cells also typically display a characteristic pattern of DNA fragmentation, that appears as a DNA "ladder" on agarose gel electrophoresis, first described in apoptotic thymocytes (Wyllie 1980). This was shown to be a result of nucleosomal cleavage by caspase-dependent deoxyribonuclease (CAD), a protein released by the cleavage of the inhibitor of CAD (ICAD) by caspases (Enari, Sakahira et al. 1998). Many of these changes are now used as markers of apoptosis, although the molecular mechanisms and features of apoptotic cell death may also be found in cells undergoing other modes of death.

2.3. Stages of apoptosis

The process of apoptotic cell death can be broadly split into three phases:

1. Initiation: the cell receives extracellular or intracellular death signals and the decision to proceed through apoptosis is made
2. Execution: the cell undergoes the process of dying, with careful dismantling of cellular machinery and scaffolding, and packaging up into apoptotic bodies

3. Disposal: the apoptotic cell and apoptotic blebs are phagocytosed in a non-inflammatory manner.

The initiation of apoptosis usually occurs via two main pathways: the death receptor pathway or the mitochondrial pathway. The death receptor pathway is typified by the TNF-α and Fas/Fas-ligand pathways, and typically involves the cleavage of caspase-8 (Wajant 2003). This may go on to activate the mitochondrial pathway secondarily, or this may be bypassed completely. Intracellular triggers of apoptosis include damage to DNA or paralysis of cellular machinery essential to the cell, by chemical or physical agents (Parone, Priault et al. 2003). These result in the activation of the mitochondrial pathway, which results in the release of cytochrome c and the subsequent formation of the apoptosome, a complex consisting of cytochrome c, APAF-1, and pro-caspase-9. This results in the cleavage of caspase-9 which then in turn activates other caspases.

The decision to initiate apoptosis also depends on the balance of pro-apoptotic and anti-apoptotic members of the bcl-2 family of proteins within the cell. These are thought to regulate the release of cytochrome c from the mitochondria, and may homo- or hetero-dimerise, and thus influence the activity of one another. Anti-apoptotic proteins, such as bcl-2 itself, may be up-regulated when the cell induces a survival response, and are often found to be present in excess in tumour cells, thus raising the cell's intrinsic resistance to chemotherapeutics.

The execution phase of apoptosis occurs once the so-called execution caspases have been activated, usually by an up-stream caspase. This cascade, once activated, usually results in the conversion of the entire pool of caspases and thus commits the cell to die; however, there are a plethora of control points along this pathway, and it is not clear where the "point-of-no-return" occurs, where the cell cannot reverse the decision to die. In neurons, caspase-3 appears to be central to the execution of apoptosis: for example, caspase-3 activation has been found in dying neurons in areas of active

pathology in EAE, in both optic tract and following relapse in the spinal cord (Ahmed, Doward et al. 2002; Hobom, Storch et al. 2004). Interestingly, CSF from patients with MS causes neuronal apoptosis in vitro, and caspase-3 inhibitors have been shown to block this process (Cid, Alvarez-Cermeno et al. 2003). In other models of neuronal cell death, including those induced by excitotoxins, caspase-3 has also been shown to play a role (Gilliams-Francis, Quaye et al. 2003), and on the basis of such findings it has been suggested that caspase-3 inhibitors may have a role as neuroprotective therapeutics (Robertson, Crocker et al. 2000).

Once the cell has committed to die, it is important that the cellular corpse is disposed of promptly, since the cell will eventually lose membrane integrity and release pro-inflammatory mediators into its environment. Phagocytes such as macrophages and microglia remove debris in a non-inflammatory manner (Fadok, Bratton et al. 1998). The apoptotic cell signals to the phagocyte through changes in its membrane, including the externalisation of phospatidylserine, a phospholipid normally present on the inner aspect of the plasma membrane that is "flipped" enzymatically to the outer leaflet during the early stages of apoptosis (Fadok, Voelker et al. 1992; Bratton, Fadok et al. 1997). Macrophages have phosphatidylserine receptors which allow them to recognise and engulf apoptotic cells (Li, Sarkisian et al. 2003). Numerous other recognition molecules exist, including components of C (Mevorach, Mascarenhas et al. 1998), that act as "eat me" signals, and recently it has been demonstrated that "don't eat me" signals are also important, such as CD31(Brown, Heinisch et al. 2002). The large array of signals a dying cell gives to the phagocyte has been suggested to confer the "meaning" of cell death: the phagocyte is able to distinguish the context in which apoptosis has occurred, for example due to infection or as part of development, and respond accordingly (Savill and Fadok 2000). Thus phagocytosis is a crucial process in not only because it limits inflammation by removing potentially toxic debris, but also because it results in modulation of the immune response appropriate to the situation it is acting within.

3. The C System

3.1. The C System: an overview

C is a central part of the innate immune system, on the front line in defence of the body from invading pathogens and in clearance of potentially damaging debris. From its earliest description over a century ago, C has been inextricably linked with cell death (Morgan 1990). The C system was first identified as a heat sensitive fraction of serum that acted in concert with antibody to kill bacteria (Ehrlich and Morgenroth 1899). A century on, research in the field is flourishing, and the fruits of the work are influencing clinical practice, with a number of therapeutics designed to modulate C activity currently undergoing clinical trials (Kirschfink 2001).

3.1.1. Function

Of course, C does more than just kill cells. It has varied and wide-ranging functions that include a crucial role in the efficient phagocytosis of pathogens and cellular debris by opsonising them with molecules such as C3b (Walport 2001). By doing so it aids the solubilisation and clearance of immune complexes that would otherwise lodge in capillary beds and cause damage. Another of its important functions is to act as a stimulus to inflammation, and here the small anaphylatoxic fragments C5a and C3a are involved by directly activating cells bearing the appropriate receptors. More recently, an important role for C in linking innate and adaptive immunity has been revealed, for example by contributing a second signal to B lymphocytes that have recognised a C opsonised antigen (Barrington, Zhang et al. 2001).

3.1.2. Structure

The C system itself is remarkable in its simplicity. Unfortunately, this simplicity is lost in a quagmire of 'difficult' terminology, a relic of history that has never been adequately addressed. Once over the terminology hurdle, the system reveals its true nature (see Figure 1.1). It consists of three recognition systems that permit identification of appropriate targets, several enzyme complexes that greatly amplify a stimulus and produce inflammatory mediators and opsonins, and a single terminal pathway that results in the formation of the MAC (Podack and Tschopp 1984; Walport 2001). The MAC is the main perpetrator in the classical story of C-mediated cell death, known for blasting holes in the cell membrane.

3.1.3. Problems

Although C is designed to protect us, it sometimes does just the opposite. Bacteria and other organisms are efficiently targeted by C, but with such a powerful pro-inflammatory system being activated in the body, there is the potential for considerable damage from friendly fire (Morgan 1990). The body therefore has many C regulators (CReg), both fluid phase and membrane bound, to minimise the chances of this occurring (Morgan and Harris 1999). Despite these defences, C still causes damage to self and has been implicated in the pathogenesis of a large number of inflammatory and immunological diseases, including rheumatoid arthritis (Linton and Morgan 1999), glomerulonephritis (Welch 2002), and multiple sclerosis (Ffrench-Constant 1994; Storch, Piddlesden et al. 1998). Although not necessarily an initiating factor in these conditions, it is often responsible for promoting and perpetuating inflammation. Cell death is often seen in these conditions, but it is not at all clear that lysis is a dominant feature in the *in vivo* activities of C.

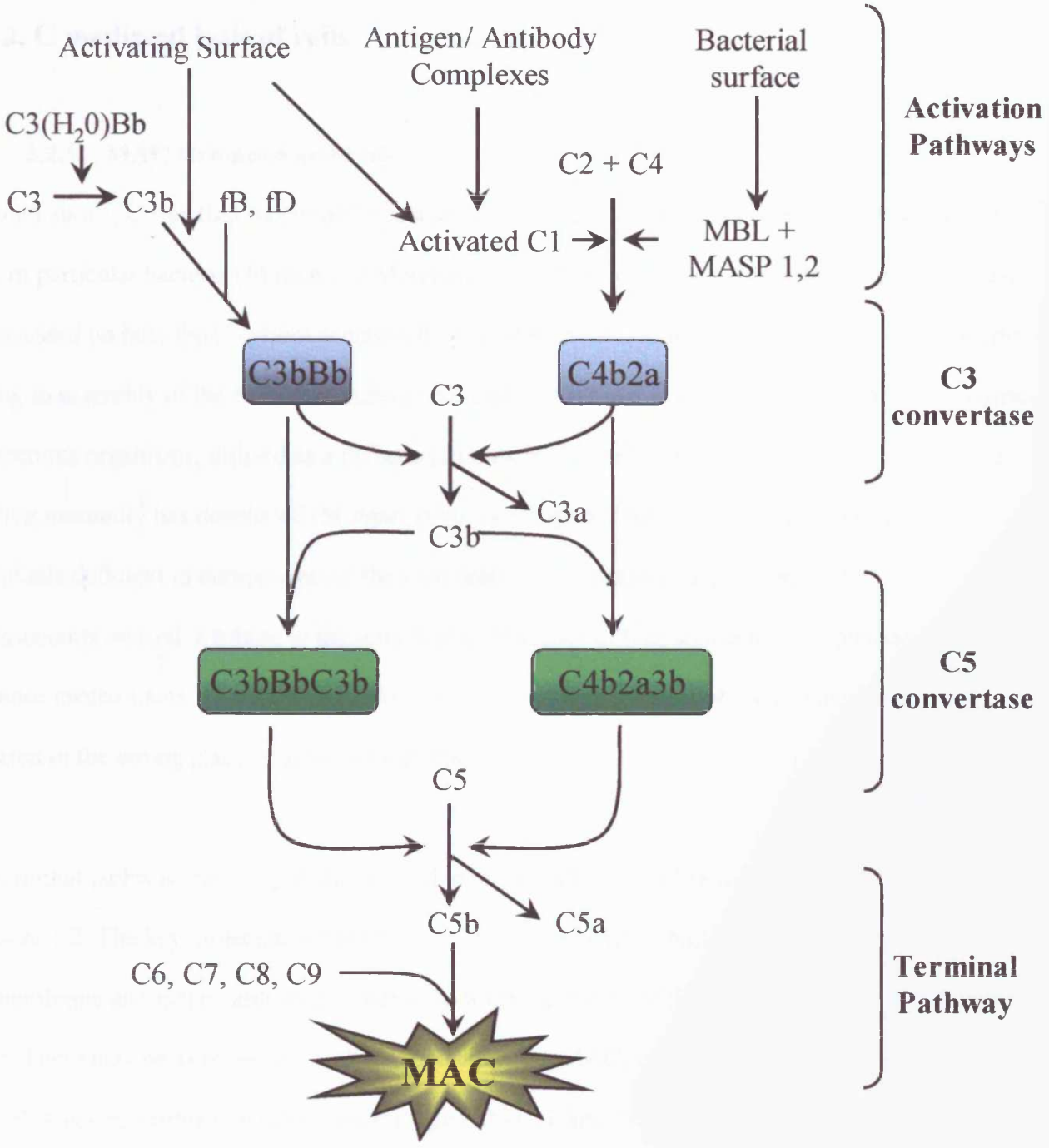


Figure 1.1. The C cascade. Activation occurs via the classical, alternative and lectin pathways, which act to target the C system. This results in the formation of the C3 and then the C5 convertases, which amplify the response and also release the anaphylatoxins C3a and C5a. The terminal pathway results in the formation of the MAC. MBL: mannan binding lectin. MASP: MBL associated serine protease.

3.2. C mediated lysis of cells

3.2.1. MAC formation and lysis

As noted above, C was first recognised because of its capacity, in conjunction with antibody, to lyse cells, in particular bacteria (Ehrlich and Morgenroth 1899; Bordet 1900). Since then, much attention has focussed on how the C system achieves this, notably the dissection of the molecular mechanisms leading to assembly of the MAC and subsequent cell death. Lysis is an important part of the response to infectious organisms, utilised as a defence not only in the early stages of infection, but also once adaptive immunity has developed (Morgan 1990; Barrington, Zhang et al. 2001). Despite this, individuals deficient in components of the membrane attack pathway are not markedly immunocompromised, a tribute to the remarkable efficiency of C opsonisation and phagocyte clearance mechanisms. However, the MAC has been blamed for much of the damage done when C is activated in the wrong place or at the wrong time.

The terminal pathway, resulting in the formation of the MAC and subsequent cell lysis is illustrated in Figure 1.2. The key molecule in the MAC is C9, an amphipathic molecule that inserts through the cell membrane and is then able to polymerise to form the "pore" of the MAC (Podack and Tschopp 1984). There may be as many as 16-18 C9 molecules per MAC, which yield the well-known tubular channel structure visible in electron micrographs (Podack and Tschopp 1984; Bhakdi and Tranum-Jensen 1991). However, only one or two molecules of C9 are required to form functional pores, and these smaller complexes do not form tubular structures. This has caused some controversy regarding how the MAC interacts with the membrane to cause lysis. Some investigators have favoured a 'leaky patch' hypothesis, where the MAC disrupts the order of the target membrane but does not form true, discrete pores (Esser 1991). Others have argued that the MAC does form pores, but when C9 is present in low copy numbers these are functional rather than rigid, hollow protein-lined channels

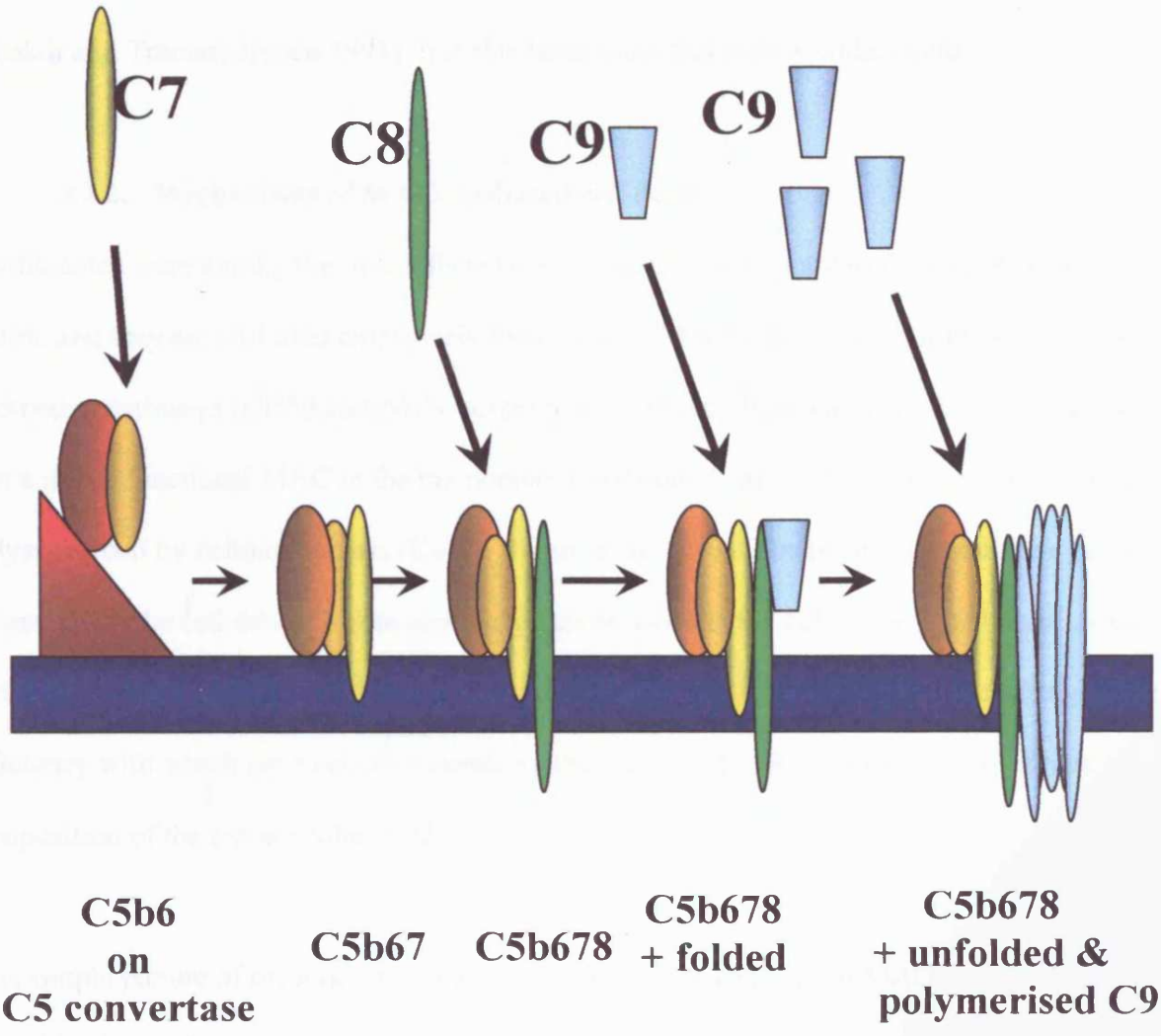


Figure 1.2. The formation of the MAC. C5b binds C6 while still bound to the C5 convertase. On binding C7 it is released and inserts into the membrane. C8 then inserts, traversing the membrane. C9 binds initially in its unfolded state, and then unfolds and polymerises to form the MAC pore.

(Bhakdi and Tranum-Jensen 1991). It is this latter view that is now widely held.

3.2.2. Mechanisms of MAC-mediated cell death

Erythrocytes were among the first cells to be investigated with regard to the lytic mechanism of C action, and they are still used extensively today in the C haemolytic assays of the classical and alternative pathways (CH50 and AH50 respectively) (Porcel, Peakman et al. 1993). It has been shown that a single functional MAC in the membrane of a metabolically inert, aged erythrocyte is sufficient to lyse the cell by colloid osmosis (Koski, Ramm et al. 1983). The breached membrane permits entry of water into the cell driven by the osmotic gradient, causing the cell to swell and burst. A similar picture occurs when liposomes are attacked (Campbell, Patel et al. 1989). Not surprisingly, the efficiency with which lysis occurs depends on the number of MACs in the cell membrane, and on the composition of the extracellular fluid.

This simple picture of osmotic cell lysis does not extend to analyses of MAC killing of nucleated cells. Metabolically active nucleated cells are, in general, much more resistant to the lytic effects of C when compared to erythrocytes (Morgan 1989). C lysis of nucleated cells displays "multi-hit" kinetics implying a requirement for many MACs on the cell surface, and factors other than colloid osmotic dysregulation, notably the presence of calcium in the extracellular fluid, influence the efficiency of killing (Campbell, Daw et al. 1981; Koski, Ramm et al. 1983). In general, nucleated cells differ from aged erythrocytes in that they possess a variety of protective mechanisms that restrict C-mediated lysis. These include ion pumps that can compensate for membrane pores and the capacity to remove MACs from the cell surface (Morgan 1989; Scolding, Morgan et al. 1989).

Calcium plays a crucial role in deciding the fate of a cell attacked by C. The earliest detectable event following MAC attack is a large influx of calcium into the cell (Morgan, Luzio et al. 1986; Sala-

Newby, Taylor et al. 1998), and increasing levels of extracellular calcium speed the progression to cell death (Kim, Carney et al. 1987). The ensuing crisis is centred on the mitochondrion. Excess calcium causes loss of the mitochondrial transmembrane potential, resulting in an energy crisis in the cell as energy-consuming ion pumps frantically try to redress the balance (Papadimitriou, Phelps et al. 1994). This precarious situation is further exacerbated by the loss of ATP and its precursors via the MAC pore into the extracellular environment (Papadimitriou, Ramm et al. 1991). The reduction in ATP through consumption and leakage renders the cell incapable of sustaining its essential metabolic processes, leading to necrosis.

3.2.3. Defence against micro-organisms

As indicated above, the MAC is not essential for the killing of most bacteria. Gram positive bacteria possess an efficient MAC avoidance strategy. The thick cell wall characteristic of these organisms prevents the MAC from reaching and breaching the inner plasma membrane of the bacterium (Morgan 1990). MAC deposition on the cell wall is without consequence. Gram negative organisms lack this thick protective coat. C activation first permeabilises the outer membrane, then aids degradation of the thin cell wall and finally exposes the inner membrane to MAC formation and lysis. MAC may also target the zones of apposition of inner and outer membranes and breach both at the same time (Born and Bhakdi 1986; Bhakdi, Kuller et al. 1987). Lysis by MAC is a major route for killing of Gram negative organisms of the genus *Neisseria* and, as a consequence, individuals deficient in the components of the MAC, such as C6 and C7, have markedly increased susceptibility to infections by *Neisseria* species that frequently cause meningitis (Morgan and Walport 1991).

3.3. Cell survival in the face of C membrane attack

3.3.1. C regulation and induced protection

We have already noted that metabolically active nucleated cells are more resistant to C attack than erythrocytes due to the presence of ion pumps and mechanisms for removal of MAC (Koski, Ramm et al. 1983; Morgan 1989). Additional protection is provided by the presence of membrane bound CReg (Morgan and Harris 1999). Cells are protected from MAC formation by a membrane bound molecule, CD59, that blocks assembly of the lytic pore (Meri, Morgan et al. 1990). In addition, nucleated cells may express ecto-proteases on their surface that can cleave C components, or ecto-kinases that can inactivate them by phosphorylation (Jurianz, Ziegler et al. 1999). Finally, when C is activated on cells, surviving cells can become protected against subsequent attack (Reiter, Ciobotariu et al. 1992; Jurianz, Ziegler et al. 1999). The mechanisms of this "induced protection" phenomenon are uncertain although it has been shown to depend upon RNA and protein synthesis (Reiter, Ciobotariu et al. 1992). A protein complex known as the large C induced protein (L-CIP), related to heat shock proteins, has been shown to be induced, which translocates to the cell membrane, although a protective function of L-CIP has not been shown (Reiter and Fishelson 1992; Fishelson, Hochman et al. 2001). "Induced protection" is not limited to MAC attack. Non-lethal amounts of the MAC protect also against other pore formers such as perforin, melittin and streptolysin O which, in turn, can induce protection from MAC attack (Reiter, Ciobotariu et al. 1995). All these protective mechanisms are important in limiting damage to host cells in areas of inflammation, but may also be put to more sinister purpose, for example when they are utilised by tumour cells in order to evade C killing (Jurianz, Ziegler et al. 1999).

3.3.2. Shedding of the MAC

Removal of MACs represents an important mechanism of cell resistance to, and recovery from, C

attack and is also one of the best defined of the non-lethal effects of MAC assembly. MACs are removed either by shedding on membrane vesicles (ectocytosis) or internalisation and degradation, depending on the cell type (Carney, Koski et al. 1985; Morgan, Dankert et al. 1987; Scolding, Morgan et al. 1989). The efficiency with which this occurs is temperature dependent (Morgan 1989). Signalling of MAC removal has been studied and again, calcium is implicated, acting in its well known capacity as a second messenger (Kraus and Fishelson 2000). Calcium influx occurs via the pore, but even in the absence of extracellular calcium, MAC still induces an increase in intracellular calcium by triggering calcium release from stores (Morgan and Campbell 1985). Protein kinase C activation occurs both directly triggered by MAC and signalled by calcium. Events further downstream are poorly defined and the precise mechanism of MAC shedding is unknown. Calcium therefore plays a double role, important in protecting cells when the MAC is present in non-lytic doses, but contributing to cell death when damage is more extensive (Morgan, Luzio et al. 1986).

3.3.3. C evasion in neurological disease

Cells present in the CNS also possess mechanisms to resist C attack, since the brain can produce a fully functional C system (Gasque, Julen et al. 1992; Gasque, Ischenko et al. 1993; Gasque, Fontaine et al. 1995; Thomas, Gasque et al. 2000). All cell types express CReg, but the levels present on the cell surface vary according to cell type and brain region (Singhrao, Neal et al. 1999). Microglia are particularly well protected, with high levels of CD55, CD46 and CD59, and up-regulate these on activation, for example following exposure to interferon-γ (Gasque, Fontaine et al. 1995); astrocytes also express these regulators, but at a lower level (Spiller, Moretto et al. 1996; Singhrao, Neal et al. 1999). Oligodendrocytes and neurons are less well protected, having lower levels of CReg and lack one or other of the activation pathway CReg – oligodendrocytes lack CD46 (Scolding, Morgan et al. 1998), and neurons lack CD55 (Singhrao, Neal et al. 2000). In addition, neurons have the unusual property of activating the classical pathway without the need for antibody, and this, coupled with

their relatively low levels of CReg, makes them more sensitive to the lytic effects of C than other CNS cell types (Singhrao, Neal et al. 2000). Rat oligodendrocytes also display this property (Scolding, Morgan et al. 1989), but this does not appear to extend to human oligodendrocytes (Zajicek, Wing et al. 1995). The importance of CReg in defending self-cells from damage in CNS disease is underlined by the increased damage seen in the CNS in animal models of disease where CReg are knocked out. For example, EAE is much more severe in CD59a knockout mice, with more extensive demyelination and axonal damage compared to wild-type animals (Mead, Neal et al. 2004).

Levels of CReg may be altered under pathological conditions in the CNS. As already noted, inflammatory mediators may up-regulate expression of CReg on glial cells in vitro, and CD55 has been found to be up-regulated during chronic, but not acute, inflammation in EAE (van Beek, van Meurs et al. 2005). Interestingly, CD59 levels have been found to be low in brains from patients with AD, particularly in areas of active pathology, suggesting that these areas may be more susceptible to MAC-induced damage (Yang, Li et al. 2000). However, the cause of the observed loss of CD59 has not been elucidated to date.

In CNS diseases where C activation is a feature of pathology, cells also attempt to protect themselves via other mechanisms. Oligodendrocytes, for example, have been shown to shed MAC on vesicles, and these have been found in the CSF of patients with MS (Scolding, Morgan et al. 1989). Sublytic doses of MAC have also been shown to induce oscillatory calcium transients in oligodendrocytes, and are associated with cell recovery (Wood, Wing et al. 1993), although the underlying mechanisms of this phenomenon are not known. Little is known about whether CNS cells display "induced protection" against C attack in the manner observed in leukaemia cell lines, or whether other mechanisms of C evasion are utilised by brain cells.

3.4. The non-lytic consequences of C membrane attack

3.4.1. Pro-inflammatory effects

MAC has been implicated in a variety of other non-lytic effects that differ according to the nature of the target cell and the system interrogated. On phagocytes (neutrophils and macrophages), cell types that are intrinsically resistant to C lysis, MAC induces profound activation with production and release of inflammatory mediators such as prostaglandins, thromboxanes, leukotrienes and reactive oxygen species (Hansch, Seitz et al. 1984). These processes contribute to homeostasis by stimulating the inflammatory response and arming the host immune system to deal with invading organisms. On the other hand, these same events may be the cause of much damage. For example, glomerular epithelial cells respond to sublytic MAC by induction of cyclo-oxygenase 2 and production of prostaglandins which contribute substantially to pathology in membranous glomerulonephritis (Takano, Cybulsky et al. 2001).

3.4.2. Signalling pathways

From studies *in vitro* on diverse cell types and *in vivo* focussing on inflammatory diseases, much has been learnt about the signal transduction pathways utilised by the MAC. However, the very diversity of cells and systems studied has created a considerable complexity. Numerous signalling pathways for MAC effects, initiated by activation of calcium flux (Cybulsky, Bonventre et al. 1990; Kraus and Fishelson 2000), receptor tyrosine kinases (Cybulsky, Takano et al. 1999) and G proteins (Rus, Niculescu et al. 2001) have been described. These in turn can then activate other signalling cascades in the target cell, including mitogen activated protein kinase (MAPK), and c-Jun N-terminal kinase (JNK) pathways (Peng, Takano et al. 2002). Downstream activation of NFκB and AP-1 may result in gene transcription (Takano, Cybulsky et al. 2001). Current knowledge, though considerable, represents a patchwork of tenuously connected datasets obtained from different targets. No unifying

concept of how MAC triggers activation events has yet emerged.

Non-lytic effects of the MAC are implicated in several diseases. A role in glomerulonephritis is noted above, and recently, dilated cardiomyopathy has come under the spotlight. MAC deposits were found on apparently viable cardiac myocytes in biopsies from patients with this disease, a finding that correlated with the expression of TNF-α, a powerful pro-inflammatory cytokine and negative inotropic factor (Zwaka, Manolov et al. 2002). In relevant cell types *in vitro*, TNF-α was induced by the MAC via NFκB, providing a possible explanation for the findings *in vivo*. Many other pathogenic processes in heart disease are being traced to similar non-lytic activating effects of the MAC, including a role in vascular smooth muscle proliferation and remodelling in atherosclerosis (Niculescu and Rus 1999). The role of MAC in pathological proliferation of cells is now receiving considerable attention.

3.4.3. Cell proliferation

The MAC has been implicated as a stimulus to cell proliferation in a number of scenarios over the past ten years. *In vivo* models of mesangioproliferative glomerulonephritis, for example, have shown that mesangial cell proliferation is markedly reduced in C6 deficient animals, which cannot form the MAC, compared to controls (Brandt, Pippin et al. 1996). In other pathological states, such as atherosclerosis, MAC deposition occurs in areas where active proliferation is taking place (Niculescu and Rus 1999). From these studies it is not clear whether the MAC induces proliferation directly, or indirectly by stimulating release of growth factors from activated cells that then in turn stimulate cell division.

Work on endothelial cells has supported the latter explanation by demonstrating that platelet derived growth factor (PDGF) and basic fibroblast growth factor (bFGF) are produced when MAC is present

on the cell membrane (Benzaquen, Nicholson-Weller et al. 1994). This process is crucially regulated by CD59: when CD59 is inactivated by glycation as may occur in diabetes, endothelial cells increase their production of PDGF and bFGF (Acosta, Hettinga et al. 2000). It has been suggested that this effect contributes to the proliferative disorders seen in diabetes, such as retinopathy and nephropathy. These data also indicate that levels of functional CD59 on a cell surface may be critical not only for protecting the cell, but also in dictating its response to sublytic C attack.

In other studies using aortic smooth muscle cells (Niculescu, Badea et al. 1999) and Schwann cells (Dashiell, Rus et al. 2000), non-lethal MAC attack does indeed directly stimulate cell proliferation. Other studies have shown an increase in DNA synthesis in response to the MAC, which in replication-competent fibroblasts probably reflects increased proliferation (Halperin, Taratuska et al. 1993), but in terminally differentiated cells such as oligodendrocytes (Rus, Niculescu et al. 1996) and mesangial cells (Couser, Pippin et al. 2001) does not. Thus the MAC induces entry to the cell cycle in many cell types, but only takes cells right through to division if they are intrinsically capable of doing so. The MAC can also amplify mitogenic signals from other growth factors such as PDGF, adding a further level of complexity.

Although the MAC has been clearly implicated in pathological cell proliferation, such as occurs in atherosclerosis, physiologically this response may be important in repairing tissues following inflammation. Thus while the proliferative effects of the MAC may be damaging in the acute phase of inflammation, during resolution it may assume an important role in repair.

The data summarised above show that the MAC must now be seen in a new light: while MAC certainly can lyse cells, the non-lytic effects may be of much greater physiological and pathological relevance (summarised in Figure 1.3). MAC can promote inflammation, increase the resistance of

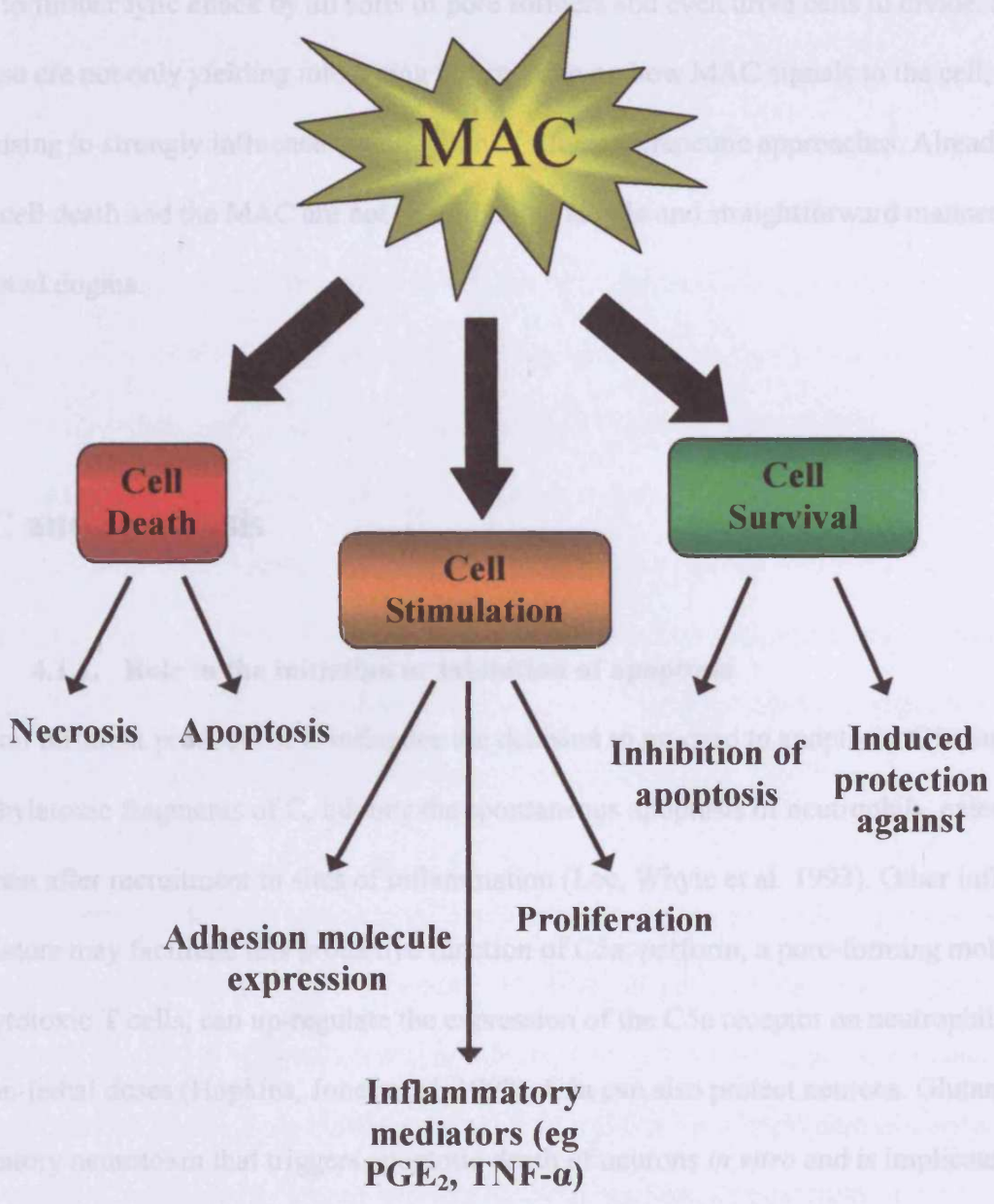


Figure 1.3. The range of cellular effects of the MAC. The combination of effects observed depends on the cell type and other environmental factors.

cells to further lytic attack by all sorts of pore formers and even drive cells to divide. Studies in disease are not only yielding interesting information on how MAC signals to the cell, but also promising to strongly influence the direction of future therapeutic approaches. Already, it is clear that lytic cell death and the MAC are not related in the simple and straightforward manner that was the accepted dogma.

4. C and apoptosis

4.1.1. Role in the initiation or inhibition of apoptosis

Several different products of C influence the decision to proceed to apoptosis. C5a, one of the small anaphylatoxic fragments of C, inhibits the spontaneous apoptosis of neutrophils, extending their lifespan after recruitment to sites of inflammation (Lee, Whyte et al. 1993). Other inflammatory mediators may facilitate this protective function of C5a: perforin, a pore-forming molecule produced by cytotoxic T cells, can up-regulate the expression of the C5a receptor on neutrophils when present in non-lethal doses (Hopkins, Jones et al. 1998). C5a can also protect neurons. Glutamate is an excitatory neurotoxin that triggers apoptotic death of neurons *in vitro* and is implicated in neuronal death in both AD and MS (Pitt, Werner et al. 2000). In a model of excitotoxic neuronal death, C5a inhibited apoptosis via inhibition of caspase 3 activity (Mukherjee and Pasinetti 2001). Confusingly, in a related system, C5a was found to increase apoptosis of a neuronal cell line (Farkas, Baranyi et al. 1998). The anaphylatoxin C3a has also been shown to protect neurones *in vitro* from apoptosis induced by the excitotoxic agent NMDA (van Beek, Nicole et al. 2001). Interestingly, in stroke the C3a receptor is up-regulated on neurons, and it is tempting to speculate that this may aid cell survival (Van Beek, Bernaudin et al. 2000).

C5a plays an important role in sepsis, where a massive systemic inflammatory response occurs that can result in multi-organ failure and death (Huber-Lang, Sarma et al. 2001). One of its features is a catastrophic dysregulation of the immune response. Immunosuppresion occurs in part as a result of immune cells undergoing apoptosis and C5a is implicated as a trigger. Thymocytes undergo intense apoptosis in the caecal ligation and puncture model of sepsis (Guo, Huber-Lang et al. 2000; Riedemann, Guo et al. 2002). Prior to the onset of apoptosis, thymocytes up-regulate C5a receptor expression in response to lipopolysaccaride and IL-6 (Riedemann, Guo et al. 2002). When these cells are then exposed to C5a, they undergo apoptosis in a caspase dependent manner (see Figure 4). This does not happen to thymocytes with a normal C5a receptor density, and therefore is only seen in the acute septic situation. Importantly, this effect may be blocked with anti-C5a antibodies, a treatment that is still effective in the model even after the onset of sepsis when most other therapies are without effect (Huber-Lang, Sarma et al. 2001; Huber-Lang, Sarma et al. 2001).

The MAC, among its many non-lytic effects, also plays a role in modulating apoptosis; the proposed signalling pathways are presented in Figure 1.4. Here, diseases of the nervous system again provide good examples. The primary target cell in MS is the oligodendrocyte, and demyelination is the hallmark of the disease (Rus, Niculescu et al. 2001). C has been implicated as a pathogenic factor in MS, and MAC-mediated killing of oligodendrocytes has been demonstrated *in vitro*. But far from being destructive, at non-lytic doses the MAC has been found to promote survival of oligodendrocytes (Soane, Rus et al. 1999). The MAC inhibits apoptosis by increasing Bcl-2 transcription and suppressing the activation of caspase-3. More recently, *in vivo* studies using DNA microarrays have shown that the MAC upregulates anti-apoptotic genes, and down regulates pro-apoptotic genes in the central nervous system in the rodent MS model, EAE (Rus, Weerth et al. 2002), providing some confirmation of the relevance of this effect. But as we have already seen, the consequences of non-lytic membrane attack go further than this. MAC can reverse the differentiated

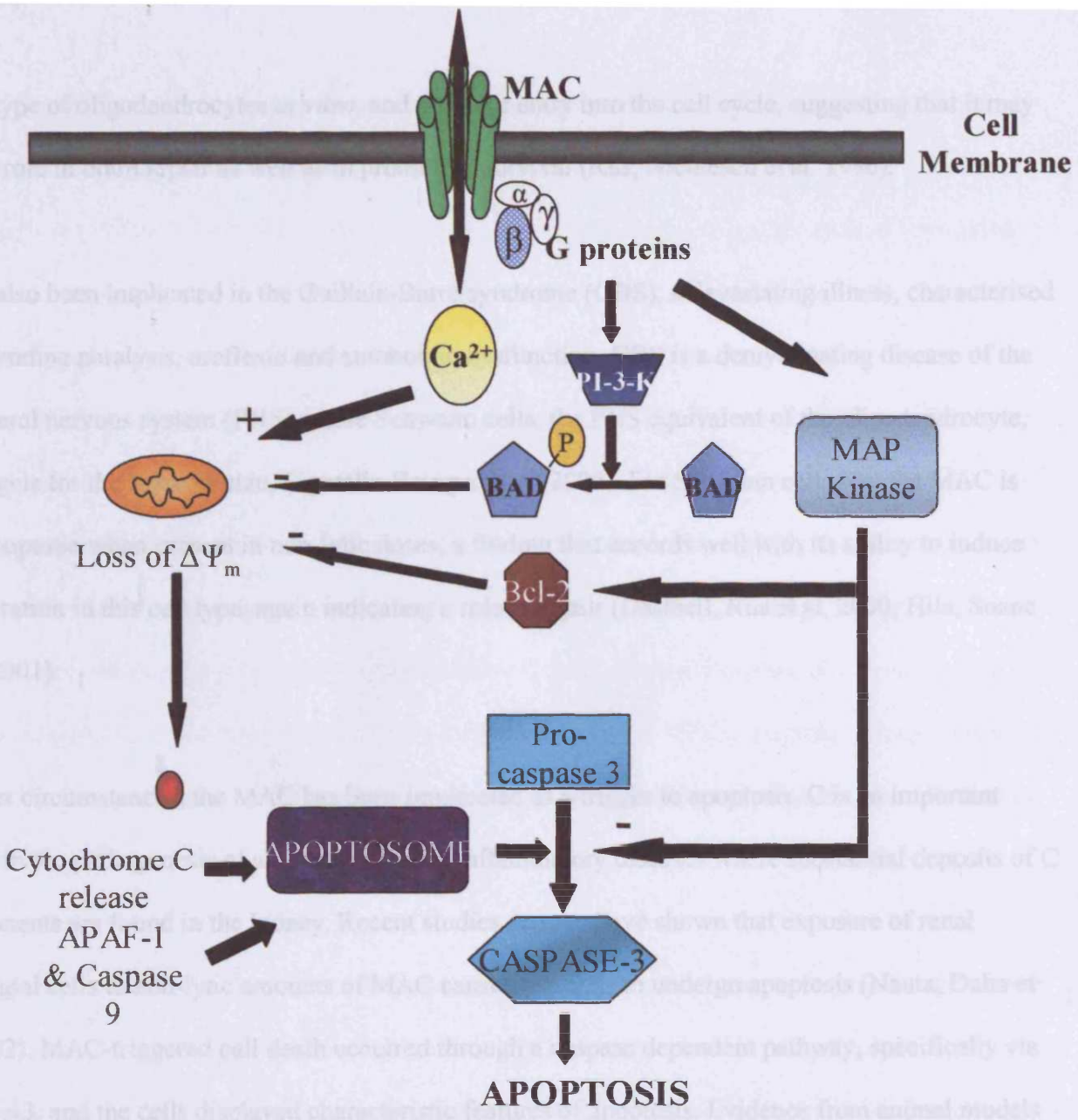


Figure 1.4. Pathways implicated in the modulation of apoptosis by MAC. The induction of apoptosis by the MAC is proposed to involve calcium influx that causes loss of the mitochondrial transmembrane potential ($\Delta\Psi m$). This results in the release of cytochrome c, which forms the apoptosome with APAF-1 and caspase-9. This results in the activation of caspase-3 which then executes the rest of the apoptotic program. Inhibition may occur via G-proteins activating the MAP kinase and PI-3 kinase pathways, with an increase in bcl-2 expression and phosphorylation of BAD inhibiting apoptosis at the level of the mitochondrial membrane.

phenotype of oligodendrocytes *in vitro*, and promote entry into the cell cycle, suggesting that it may have a role in brain repair as well as in promoting survival (Rus, Niculescu et al. 1996).

C has also been implicated in the Guillain-Barre syndrome (GBS), a devastating illness, characterised by ascending paralysis, areflexia and autonomic dysfunction. GBS is a demyelinating disease of the peripheral nervous system (PNS) where Schwann cells, the PNS equivalent of the oligodendrocyte, are targets for the MAC (Putzu, Figarella-Branger et al. 2000). For Schwann cells too, the MAC is anti-apoptotic when present in non-lytic doses, a finding that accords well with its ability to induce proliferation in this cell type, again indicating a role in repair (Dashiell, Rus et al. 2000; Hila, Soane et al. 2001).

In other circumstances, the MAC has been implicated as a trigger to apoptosis. C is an important player in the pathogenesis of a number of renal inflammatory diseases where substantial deposits of C components are found in the kidney. Recent studies *in vitro* have shown that exposure of renal mesangial cells to non-lytic amounts of MAC caused the cells to undergo apoptosis (Nauta, Daha et al. 2002). MAC-triggered cell death occurred through a caspase dependent pathway, specifically via caspase-3, and the cells displayed characteristic features of apoptosis. Evidence from animal models of renal disease also implicates the MAC in triggering apoptosis. In antibody-dependent glomerulonephritis models, such as those induced by concavalin A or anti-Thy1.1, the abundant glomerular cell apoptosis seen in normal rats was much reduced in C6 deficient rats, where the MAC cannot form (Sato, Van Dixhoorn et al. 1999; Hughes, Nangaku et al. 2000).

C has been implicated in cell death in many models of ischaemia-reperfusion injury, for example, myocardial infarction and stroke (D'Ambrosio, Pinsky et al. 2001; Monsinjon, Richard et al. 2001). Cell death occurs via apoptosis as well as necrosis in these injuries (Vakeva, Agah et al. 1998) and

the MAC contributes to both. In a myocardial ischaemia/reperfusion model, anti-C5 antibodies inhibited damage following reperfusion and markedly reduced the number of apoptotic cells in the myocardium (Vakeva, Agah et al. 1998). Thus blocking MAC and C5a by this method attenuated both lysis and apoptosis, providing an exciting avenue for improving the efficacy of thrombolytic therapy. Importantly, apoptosis has been found to occur prior to the onset of necrosis in a renal model of ischaemia/ reperfusion injury (Daemen, van 't Veer et al. 1999). When apoptosis was inhibited by caspase inhibitors or by survival factors such as IGF-1, inflammation was prevented and renal damage markedly diminished. These results are, at first sight, counter-intuitive in that apoptotic cell death is considered to be non-inflammatory. However, there is evidence that apoptosis can in some circumstances exacerbate inflammation and here too C is implicated. For example, in an *in vitro* model of apoptosis in human umbilical vein endothelial cells (HUVEC), apoptotic cells activated the classical pathway resulting in C3 deposition and release of pro-inflammatory molecules (Tsuji, Kaji et al. 1994).

A note of caution must be injected at this point. Studies of C-triggered apoptosis usually rely heavily on the observation of the typical morphological changes of apoptosis. However, C attacked cells may undergo a type of death that has nuclear features of apoptosis, such as nucleosomal fragmentation, but cytoplasmic features of necrosis, such as swelling and disruption of organelles (Shimizu, Masuda et al. 2000). It has been suggested that this represents another form of cell death, 'apoptotic necrosis'. Such features have also been noted and investigated *in vitro* and have shown that such death is primarily necrotic but with secondary features that resemble nuclear apoptosis, most likely due to an extracellular DNase entering through the disrupted membrane (Cragg, Howatt et al. 2000). Studies that identify apoptosis by DNA fragmentation and TUNEL assay may therefore be misleading.

4.1.2. C-mediated recognition and clearance of apoptotic cells

C is also involved in the clearance of apoptotic cells. The safe clearance of cells dying by apoptosis is essential to prevent an inflammatory response (Fadok, Bratton et al. 1998; Savill and Fadok 2000). If apoptotic cells are not efficiently cleared, they undergo secondary necrosis, releasing pro-inflammatory mediators into the environment. Most apoptotic cells are cleared by professional phagocytes such as macrophages, although they can be cleared by other cells, albeit less efficiently. Numerous cell surface features of apoptotic cells have been implicated in recognition by phagocytes (Savill and Fadok 2000; Ogden, deCathelineau et al. 2001; Nauta, Raaschou-Jensen et al. 2002). The contribution of C had received surprisingly little attention until quite recently (Fishelson, Attali et al. 2001). The surface blebs that are characteristic of cells undergoing apoptosis were shown to bind C1q through the globular head domains, a conformation that permits activation of the classical pathway and deposition of other C fragments (Korb and Ahearn 1997). C1q binds specific receptors on the phagocyte surface, including CD91 and calreticulin, to initiate phagocytosis (Ogden, deCathelineau et al. 2001). Mannose binding lectin (MBL), the lectin pathway analogue of C1q, also binds apoptotic cells and recruits phagocytes through these same receptors. Apoptotic cells may also directly activate the alternative pathway (Tsuji, Kaji et al. 1994), and all three pathways may result in C3 deposition and activation of the terminal pathway. Fragments of C4 and C3 deposited on the apoptotic cells will bind the phagocyte-expressed receptors CR1, CR3 and CR4 to further aid recognition and clearance (Takizawa, Tsuji et al. 1996). Binding through CR3 also signals the phagocyte to down-regulate IL-12 and interferon-γ secretion. This response has an additional anti-inflammatory effect by dampening cell-mediated immunity (Marth and Kelsall 1997). From such studies, it has become increasingly clear that phagocytes respond to C and other apoptotic cell surface signals in the context of their environment, thus defining the 'meaning' of cell death. Phagocytes may respond by altering the susceptibility of neighbouring cells to death, or by modulating the inflammatory response (Savill and Fadok 2000).

The relevance of C for apoptosis is clearly evident in systemic lupus erythematosus (SLE), a systemic autoimmune disorder characterised by autoantibodies to nuclear antigens(Walport 2001). The most obvious role that C plays in this disease is in causing tissue damage when activated by immune complexes deposited in organs such as the kidneys (Walport 2001). This results in inflammation in the kidney, brain, and other affected organs. However, C deficiencies, particularly those of the classical pathway, also predispose to pathology resembling SLE, a finding that appears at first to weaken the case for C involvement (Taylor, Carugati et al. 2000; Botto 2001). The association with SLE is seen most strikingly in C1q deficiency, the strongest single gene association with SLE, and to a lesser extent with C4 and C2 deficiency (Taylor, Carugati et al. 2000), and can be explained by the roles noted above for C1q and other C components in clearing apoptotic cells. This is evident in C1q deficient mice, where multiple apoptotic bodies were seen in the kidney in association with a glomerulonephritis similar to that seen in SLE (Botto, Dell'Agnola et al. 1998). Even in the absence of C deficiency, SLE is associated with C consumption and with antibodies against C1q, both of which predict severe disease (Moroni, Trendelenburg et al. 2001). This has led to the hypothesis that SLE in man and mice is caused by defects in the removal of apoptotic cell debris, the "waste disposal" hypothesis (Walport 2001). Apoptotic cells that are not cleared generate an autoimmune response because cytoplasmic and nuclear antigens that are normally sequestered become exposed at the cell surface or outside the lysed cell (Bell and Morrison 1991; Mohan, Adams et al. 1993; Fishelson, Attali et al. 2001). Apoptotic cells undergoing secondary necrosis may also provide 'danger' signals to the antigen presenting cells and T cells in the area, converting the response to these antigens from tolerogenic to immunogenic (Savill and Fadok 2000). Activated T cells can then stimulate autoreactive B cells to differentiate into plasma cells and start producing autoantibodies (Walport 2001).

The 'waste disposal' hypothesis is further supported by the observations that mice deficient in serum

amyloid P (SAP) or DNase 1, both involved in clearance of apoptotic debris, also develop an SLE-like disease (Bickerstaff, Botto et al. 1999; Napirei, Karsunky et al. 2000). The pentraxin SAP solubilises DNA and chromatin in the extracellular fluid and transports it to the liver where it is catabolised, while DNase1 breaks down DNA into non-antigenic fragments. Indeed, SLE patients have low levels of DNase 1, adding weight to the involvement of this enzyme, and the importance of reducing the antigenicity of DNA by cleaving it (Napirei, Karsunky et al. 2000). C reactive protein (CRP), also a member of the pentraxin family and closely related to SAP, binds small nuclear ribonucleoproteins. These are also autoantigens in SLE, and it is widely recognised that disease flares are associated with a deficient CRP response, which may be associated with poor clearance of such autoantigenic material (Gershov, Kim et al. 2000).

These studies emphasise the multi-factorial process of efficient waste disposal and highlight the fact that defects in any one of the key handling processes can cause failed clearance and pathology. They also suggest that treatment strategies targeting the clearance mechanisms for apoptotic cells might be effective in many diseases.

5. Summary and Hypothesis

The interaction between the C system and the initiation of apoptosis, and C mediated clearance of dying cells, are important processes under normal physiological conditions and play a central role in many diseases. These processes occur in the brain, and C-mediated effects have been shown on oligodendrocyte cell death. However, data are lacking in regard to neurons, cells that are crucial in dictating the neurological sequelae of disease and which have a unique capacity to spontaneously activate C. CReg loss from apoptotic neurons may be of importance in either exacerbating

inflammation or in promoting clearance of these cells while leaving fluid phase regulators in the environment to help limit C activity. In addition, sublytic MAC may have protective effects on neurons, not only by inducing protection from further C attack, but possibly in protecting them from apoptotic stimuli as well.

The hypothesis for this study therefore is:

The C system modulates neuronal cell turnover in the central nervous system

The specific aims are to:

1. Define the C regulator profile of neuronal cells and changes during apoptosis
2. Determine whether sublytic MAC modulates apoptosis in neuronal cells
3. Determine whether other sublytic effects of the MAC occur on neuronal cells, including induced protection
4. Assess whether C deposition and neuronal apoptosis are correlated with disease processes in vivo

Chapter 2: Materials and Methods

1. Materials

1.1. Chemicals and buffers

Camptothecin (CPT) was obtained from Sigma (Gillingham, Dorset, UK), and C2 and C6 ceramide, and kainic acid were from Biomol (Affiniti Research Products, Exeter, UK). GM6001 and inhibitors of matrix metalloprotease (MMP)-2, MMP-3, MMP-8, MMP-9 and tissue inhibitor of metalloprotease (TIMP)-3 were from Calbiochem (Nottingham, UK). β nicotinamide dinucleotide hydrate (βNADH), sodium pyruvate, trichloroacetic acid (TCA) and aprotinin were obtained from Sigma. Calcein.AM, fura-2.AM, and zymosan-Alexa488 bioparticles were obtained from Molecular Probes (Invitrogen, Paisley, UK). Fresh frozen plasma (FFP) was obtained from the Welsh Blood Transfusion Service (Llantrisant, UK).

FACS buffer contained PBS, 1% BSA, and 5mM EDTA. Annexin binding buffer (ABB) contained 10mM HEPES, 140mM NaCl, and 2.5mM $CaCl_2$, pH 7.4 adjusted with NaOH. Lysis buffer contained 150mM NaCl, 50mM Tris, 5mM EDTA, 1% NP-40 and aprotinin (10μg/ml).

1.2. Antibodies

The monoclonal anti-CD59 antibody BRIC229 and the anti-CD55 antibody BRIC216 were from the International Blood Group Reference Laboratory (Bristol, UK), the anti-CD46 mAb MEM258 was a kind gift from Dr V. Horesji, Prague, and the anti-rat Crry mAb TLDIIIC11 was a gift of Dr Bill

Hickey. The anti-C9 neoepitope mAb B7, the pAb anti-human C9, anti-rat mAb CD55 RDIII7, anti-rat CD59 mAb 6D1 were raised in-house, and the anti-C3b mAb C3/30 was from Dr P. Taylor (Ciba, Horsham, UK). Anti-CD14-RPE, anti-C receptor 3 (CR3; CD11b) and anti-C receptor 4 (CR4; CD11c), anti-macrophage and anti-human C3c antibodies were from Dakocytomation (Ely, Cambridgeshire, UK). Anti-CD200 mAb was from BD Bioscience. Anti-PARP mAbs were from BD Biosciences and Sigma. RPE-conjugated goat anti-mouse antibody was from Dakocytomation, and donkey anti-mouse-horseradish peroxidase (HRPO) was from BioRad (Hemel Hempstead, UK).

1.3. **Cells and culture media**

IMR-32 neuroblastoma cells and B50 rat neuroblastoma cells were obtained from ATCC (Teddington, Middlesex, UK). RPMI 1640 culture medium, foetal calf serum (FCS), sodium pyruvate, streptomycin and penicillin, L-glutamine, and amphotericin were from Gibco (Paisley, UK).

1.4. **Apoptosis reagents**

CaspACE (FITC-VAD-fmk) was obtained from Promega (Southampton, UK), and the caspase inhibitors zVAD-fmk (pan-caspase) and zDEVD-fmk (caspase-3) and the TUNEL assay kit were from R&D Systems (Abingdon, UK). Annexin-V-FITC was from BD Biosciences (Oxford, UK).

1.5. **Complement reagents**

C fixation diluent (CFD) tablets were from Oxoid (Basingstoke, UK). Normal human serum (NHS) was obtained from healthy donors: blood (50-60ml) collected by venesection, transferred to glass universal containers and allowed to clot at 22°C. The clotted blood was then centrifuged at 1000g for 10 minutes, and the serum collected and stored at -80°C until use. Heat inactivated NHS (HI-NHS)

was generated from the stock of NHS by heating to 56°C for 45 minutes in a water bath.

C1q-FITC, BSA-FITC and C3/30-FITC were made by direct conjugation as described (Roederer 2004). FITC conjugation reaction buffer contained 500 mM sodium carbonate, pH 9.5, and the storage buffer contained 10mM Tris, 150mM NaCl and pHix, pH 8.2. FITC (10mg; Sigma) was dissolved in DMSO, and proteins were dialysed into FITC conjugation reaction buffer. 60μg FITC was added per 1mg protein, and incubated at 22°C in the dark on a rotamixer for 1 hour. A PD-10 gel filtration column was equilibrated with 5 volumes of storage buffer, and the FITC conjugated protein was passed over the column and 0.5ml fractions collected. Fractions were analysed for protein content using Coomassie reagent (Pierce, Perbio Science UK Ltd, Cramlington, UK), and the protein-containing fractions pooled and stored at 4°C.

1.6. **Phagocytosis assay reagents**

PKH26 red fluorescent mini linker kit and Accuspin tubes were obtained from Sigma, lymphoprep was from Axis-Shield (Upton Huntingdon, Cambs, UK) and buffy coat was from the Welsh Blood Transfusion Service.

2. Cell culture

2.1. **Neuronal cell lines**

IMR-32 human neuroblastoma cells and B50 rat neuroblastoma cells were maintained in RPMI 1640 supplemented with 5% heat-inactivated FCS (HI-FCS), sodium pyruvate (5mM), streptomycin (50U/

ml), penicillin (50 U/ml) and L-glutamine (2mM). Culture medium was replaced every 2-3 days and cells were split 1:3 to 1:6 every 4-5 days.

2.2. Isolation and differentiation of primary human macrophages

Primary human monocytes were isolated from buffy coat. Lymphoprep (15ml) was layered onto the frit in Accuspin tubes and spun at 250g for 30 seconds at 22°C. Buffy coat (30ml) was then layered over the frit and spun at 250g for 30 minutes at 22°C. The plasma layer was carefully removed, and the mononuclear cell layer, below the plasma layer, was then harvested. Cells were washed once in an equal volume of PBS/0.4% tri-sodium citrate and spun at 250g for 5 minutes at 4°C. To the pellet was added 10ml of a hypotonic saline solution (0.2%) for 30 sec on ice and then 10ml of a hypertonic saline solution (1.6%) was added and spun at 250g for 5 minutes at 4°C. The pellet was washed six times with 10ml PBS/0.4% tri-sodium citrate. After the last wash cells were re-suspended cells in 5% HI-NHS in RPMI with additives as previously described, and counted, before plating out in 12 well plates ($1x10^6$ cells per well) and placing in a CO_2 incubator at 37°C. Culture medium was replaced after two days. The cells were allowed to differentiate into macrophages for 7-14 days and used for the assay at this point.

3. C components and C deficient sera

3.1. Purification of C6

FFP was thawed, and 1mM PMSF was added. PEG 4000 (15% in dH_2O) was added to the FFP to final concentration of 5% PEG 4000 and was stirred for 30 minutes at 4^0C. The mixture was then spun at 5,500g for 20 minutes at 4^0C. The pellet was discarded and 11g PEG 4000 was added to each

100ml of supernatant, and stirred for 30 minutes at 4°C. The mixture was spun at 5,500g for 20 minutes and the supernatant discarded. The pellet was dissolved in wash buffer (PBS + 500mM NaCl) overnight at 4°C. The C6 affinity column (mAb FG-1 20mg in 10ml, raised in-house) was washed with 50-100ml of wash buffer and the sample then slowly loaded. The column was then washed with 50-100ml of wash buffer. C6 was then eluted with elution buffer (PBS + 500mM NaCl + 0.5% diethylamine) and 2ml fractions collected. The column was then washed with 50-100ml wash buffer. Fractions were assayed for protein content by adding 50µl Coomassie reagent to 50µl sample. Fractions containing protein were pooled and dialysed into PBS overnight at 4°C. The dialysed samples were then concentrated using an Amicon concentration system using a 30kDa cut-off filter.

Samples were assayed for C6 activity using rat C6 deficient serum in a classical haemolysis assay. Protein purity was assessed by SDS-PAGE and checked for C6 specificity using Western blotting. Where appropriate, gel filtration chromatography was used in a final purification step, using a Superdex 200 10/300 GL column on an AKTA FPLC (GE Healthcare, Buckinghamshire, UK). Final protein concentration was assayed using the micro BCA assay kit (Pierce).

3.2. Generation of C8 depleted serum and C9 depleted serum

The anti-C8 mAb affinity column was washed with 50-100ml CFD, and fresh NHS was run slowly over the column. Fractions (1ml) containing serum were collected. The column was then washed with 0.5% diethylamine in PBS (approx. 50ml) to regenerate the column, and then washed with 50ml PBS, followed by PBS/ 0.1% sodium azide prior to storage. Classical haemolysis assays with and without C8 were used to check depletion and fractions with good activity and minimal dilution with CFD were pooled.

C9 depleted serum was generated using an anti-C9 mAb affinity column and the same protocol as

described above.

4. Immunolabelling and flow cytometry

4.1. Immuno-labelling of cells

For immunostaining harvested cells were first washed twice in 200µl FACS buffer. Primary antibodies were diluted in FACS buffer (100µl), and incubated with 1 x 10^5 cells for 30 minutes at 22°C. Cells were then washed twice in 200µl FACS buffer, and incubated with 100µl secondary antibody (eg goat anti-mouse-RPE) in FACS buffer at 22°C in the dark for 30 minutes. Cells were washed twice in 200µl FACS buffer, resuspended in 200µl FACS buffer, and analysed on a FACScalibur flow cytometer.

4.2. Flow cytometry

Flow cytometry was carried out on a BD FACScalibur flow cytometer (BD) and analysis was carried out using CellQuest (BD) and WinMDI software (Joseph Trotter, Scripps Institute, USA).

5. SDS-PAGE and Western blotting

SDS-PAGE was performed as described (Laemmli 1970). Proteins were visualised by staining with 0.1% w/v Coomassie Brilliant Blue for 30 minutes at 22°C. Gels were destained with 10% acetic acid + 40% methanol in dH_2O until bands were visualised, and dried in gel drying buffer (40% methanol, 4% glycerol in dH_2O) between cellophane sheets.

For Western blotting, proteins separated using SDS-PAGE were transferred onto a nitrocellulose membrane and blocked using PBS/0.1% Tween-20/ 5% non-fat dried milk (10ml) for 1 hour at 22°C. Membranes were then incubated with primary antibodies overnight at 4°C in PBS/Tween/milk (10ml), and then washed 3 times in PBS/Tween (10ml) for 10 minutes on a rotamixer. Membranes were then incubated with the secondary antibody (donkey anti-mouse-HRPO) in PBS/Tween/milk (10ml) for 1 hour at room temperature and washed 3 times in PBS/Tween (10ml). Membranes were developed using the ECL Western Blotting detection system (Pierce) and imaged using Kodak Medical Imaging film.

6. Apoptosis induction and detection

6.1. Apoptosis induction

IMR-32 were plated out on 12 well plates (4 x 10^5 cells per well), or in T25 flasks (2 x 10^6 cells), or T80 flasks (6.4 x 10^6 cells) and allowed to adhere for 24 hours. Induction of apoptosis was achieved by replacing the medium with fresh RPMI 1640 with additives as above and 5% HI-FCS containing 1 μM CPT, or 60μM each of C2 and C6 ceramide, or carrier controls (either DMSO diluted 1 in 10,000 or 1% BSA respectively). In UV irradiation experiments, IMR32 were plated out in 6 well plates with 7.5 x 10^5 cells per well and allowed to adhere for 24 hours. Cells were then irradiated in a Stratalinker 2400 (Stratagene, UK) with a UV dose of 400mJ in fresh serum-free RPMI. Cells were harvested at indicated time points by harvesting the medium, briefly trypsinising the remaining cell monolayer, and adding this to the harvested medium. Cells were then spun at 300g at 22°C for 3 minutes, and washed in serum-free RPMI before incubation with the relevant apoptosis marker or antibody.

6.2. **Annexin-V-FITC and Propidium Iodide (PI) staining**

For Annexin-V-FITC staining, 1 x 10^5 cells were washed twice in 200µl ABB, followed by incubation with annexin-V-FITC according to the manufacturer's instructions (1 in 50 of standard aliquot) and 2µg/ml PI in 100µl ABB for 20 minutes at 22°C in the dark. Cells were then transferred to FACS tubes containing 100µl ABB, and analysed on a BD FACScalibur using the FL-1 and FL-2 channels.

6.3. **Fluorescent Caspase Inhibitors**

For caspACE staining, 1 x 10^5 cells were incubated in 100µl of 10µM caspACE in FACS buffer for 20 minutes at 22°C in the dark. Cells were then washed twice with 200µl FACS buffer and analysed on the FL-1 channel of a BD FACScalibur flow cytometer.

7. Complement assays

7.1. **Haemolysis assays**

Sensitised sheep erythrocytes (EA) were prepared by washing 2ml of sheep E in Alsever's buffer twice with 15ml PBS, and centrifuging at 1000g for 5 minutes. A 4% suspension of sheep E was made by adding 200µl of the E pellet to 5ml PBS. Anti-sheep immunoglobulin (Amboceptor, 20µl) was diluted in 5ml PBS and the solutions were mixed and incubated at 37°C for 30 minutes. EA were then washed twice in CFD and resuspended in 10ml CFD (2% suspension final).

For the lysis assay a 96 well round-bottomed plate was used. Dilutions of serum to be tested were prepared in CFD, NHS control, and positive control (water), and negative control (CFD only) using

100µl per well. Sheep EA (50µl) was added to each well, agitated briefly, and incubated for 60 minutes at 37°C. The plate was then centrifuged at 1800rpm for 4 minutes and 100µl of supernatant was transferred to a 96 well flat-bottomed plate containing 100µl of dH_2O per well. The plate was then read in a spectrophotometer at 415nm.

Percent lysis was calculated:

$$\% \text{ lysis} = \frac{(\text{Abs(test)} - \text{Abs(negative control)})}{(\text{Abs(positive control)} - \text{Abs(negative control)})} \times 100$$

CH50 was calculated by plotting log(% serum) vs log (y(1-y)), where y = % haemolysis, and calculating CH50 as $(1/10^{x\ \text{intercept}})$x2000.

7.2. Nucleated cell lysis assay

7.2.1. PI assay

Cells were harvested, and washed twice in CFD by centrifugation at 300g for 3 minutes at 22°C. NHS or HI-NHS (100µl) was diluted as appropriate in CFD and added to 1 x 10^5 cells and incubated for 1 hour at 37°C, 5% CO_2. Following incubation, samples were centrifuged at 300g for 3 minutes, and washed twice in FACS buffer. PI (2µg/ml) in FACS buffer was added, and cells analysed on the FL-2 channel of a BD FACScalibur flow cytometer. Lysis was defined as cells that were PI positive. C-specific lysis was calculated using the following equation:

$$\text{C-specific lysis} = \frac{\%\ \text{PI+ (NHS)} - \%\ \text{PI+ (HI-NHS)}}{100 - \%\ \text{PI+ (HI-NHS)}}$$

In some experiments, serum was diluted in RPMI instead of CFD.

7.2.2. LDH assay

Cells were harvested, and washed twice in CFD by centrifugation at 300g for 3 minutes at 22°C. NHS or HI-NHS (100µl) was diluted as appropriate in CFD and added to 1 x 10^5 cells and incubated for 1 hour at 37°C, 5% CO_2. The 100% lysis control used 0.2% triton X-100 in CFD, and for the 0% lysis control CFD alone was used. Following incubation, samples were centrifuged at 300g for 3 minutes and 50µl of the supernatant was transferred into a flat-bottomed 96 well plate. Serum-only controls were included at the same concentrations as used in the lysis assay.

For the lactate dehydrogenase (LDH) cell death assay, the following were used: solution A was 5.6mM Tris, 5.6mM EDTA, pH 7.4 (HCl), solution B was 148.5mg sodium pyruvate in 10ml dH_2O and solution C was NADH at 1.3mg/ml in solution A made up fresh just before use. To each well of the 96 well plate 100µl solution C was added. Just before analysis, 10µl solution B was added to each well. Analysis was performed on a FLUOstar Optima spectrophotometer (BMG Labtechnologies, Aylesbury, UK), taking 340nm absorbance readings every 2 minutes for 20 minutes. The rate of fall of absorbance at 340nm was calculated for each well, reflecting the decrease in amount of NADH present, and therefore the activity of LDH present, and the following equation applied to calculate % lysis:

$$\% \text{ lysis} = \frac{\text{sample} - \text{serum blank}}{100\% \text{ lysis} - \text{serum blank}} \times 100$$

In some experiments, serum was diluted in RPMI instead of CFD.

7.3. C deposition assays

Cells were harvested as described above, washed in serum-free RPMI, and 1 x 10^5 cells incubated with 100µl C8d serum or with C1q-FITC (10µg/ml) in RPMI at the required dilution in RPMI for 1 hour. Cells stained with C1q-FITC were washed twice in FACS buffer and analysed on the flow cytometer on the FL-1 channel. Cells incubated with C8d serum were washed twice in ice-cold FACS buffer and incubated with anti-C3b antibody (C3/30 mAb) on ice for 30 minutes. Cells were then washed twice in ice-cold FACS buffer, and antibody stained cells were incubated with anti-mouse-RPE for 30 minutes on ice in the dark. Cells were then washed twice in cold FACS buffer and analysed on the FL-2 channel of a flow cytometer. In some experiments, directly labelled C3/30-FITC was used in a one step staining procedure, and analysis carried out using the FL-1 channel.

To assess MAC binding, cells were subject to C lysis assay as described in 7.2, and then stained as described above, using the primary antibody B7 (anti-C9 neoepitope; 2µg/ml).

For double staining experiments, apoptosis markers were added either with the secondary antibody (caspACE), or after antibody staining and washing twice in ABB (Annexin-V-FITC) using the concentrations and incubation times described above for the apoptosis assays.

8. Statistics

Statistical analysis to compare differences between two groups was performed using the Student's *t*-test; where discontinuous variables were analysed the Mann-Whitney U test was used. For multiple group analysis, one-way ANOVA with post hoc analysis using the Tukey test was used. Results are expressed as mean +/- SEM or +/- SD as indicated in the text.

Chapter 3

Complement regulator loss from neuronal cells: mechanisms and functional consequences

1. Introduction

C activation occurs in areas of active disease in both neuroinflammation and neurodegeneration (Compston, Morgan et al. 1989; Linington, Morgan et al. 1989; Itagaki, Akiyama et al. 1994; Mead, Singhrao et al. 2002; Weerth, Rus et al. 2003). Loss of neurons is recognised as a significant component in the pathology of these diseases and in MS neuronal loss has been correlated with poor recovery of function and permanent neurological disability (Davie, Barker et al. 1995). In vitro studies have shown that neurons spontaneously activate C and are particularly susceptible to lytic damage by the MAC (Gasque, Thomas et al. 1996; Singhrao, Neal et al. 2000). In the rodent model of MS, EAE, C activation and formation of the MAC are known to cause axonal damage and neuronal loss (Mead, Singhrao et al. 2002) and therapeutic approaches involving inhibition of C in the acute phase have been shown to be neuroprotective (Piddlesden, Storch et al. 1994). However, it is now becoming evident that neuronal death in MS and EAE is largely apoptotic rather than necrotic, and in EAE, neuronal death via apoptosis has been shown to correlate with functional deficit (Dowling, Husar et al. 1997; Meyer, Weissert et al. 2001).

Apoptotic cells typically undergo a series of morphological changes, including cell shrinkage, chromatin condensation, and cell surface bleb formation, before being removed by phagocytes

(Savill, Wyllie et al. 1989). Apoptotic cells in the CNS are cleared by microglia or astrocytes in a non-inflammatory manner (Stolzing and Grune 2004). Failure to clear such apoptotic debris may provoke a damaging inflammatory reaction and exacerbate pathology, since dying cells eventually undergo secondary necrosis and release pro-inflammatory mediators into the environment. Recognition is mediated by changes in molecules expressed at the surface of the dying cell, such as exposure of phosphatidylserine (Savill and Fadok 2000; Li, Sarkisian et al. 2003), and by the binding of various components and activation products of the C system.

C has been shown to play a role in clearing apoptotic debris in many tissues through binding of C1q and C3b (Botto, Dell'Agnola et al. 1998; Mevorach, Mascarenhas et al. 1998; Taylor, Carugati et al. 2000). This appears to occur late in the apoptotic process suggesting that it may act as a fail-safe mechanism (Gaipl, Kuenkele et al. 2001; Zwart, Ciurana et al. 2004). However, C is also known to be pro-inflammatory and to cause necrotic cell death by insertion of the MAC (Koski, Ramm et al. 1983). The balance between the clearance enhancing anti-inflammatory properties of C and its necrosis-inducing pro-inflammatory actions may be crucial in determining the consequences of apoptotic cell death in tissues. Self-cells are normally protected from C activation by the membrane CRegs CD46 and CD55 controlling the C3 convertase, and CD59 controlling the MAC (Morgan and Harris 1999).

Changes in CReg during neuronal apoptosis may be extremely important under pathological conditions, since loss of CReg may leave dying neurons stripped of protection against C, and coupled with their capacity to spontaneously activate C (Singhrao, Neal et al. 2000), apoptotic neurons could become potent catalysts for uncontrolled C activation and become increasingly prone to C-mediated lysis. However, increased C activation and deposition of C3 fragments could also result in brisk opsonisation and subsequent clearance by phagocytes, avoiding any worsening of the inflammatory response. C activation may therefore be a critical factor in deciding the fate of

apoptotic neurons and in determining whether inflammation is provoked or inhibited in the brain.

In view of the largely protective responses normally associated with apoptosis, it was hypothesized that:

C activation on apoptotic neuronal cells is predominantly anti-inflammatory.

The aims were therefore to identify the factors that influence C activation on apoptotic neuronal cells, and study the consequences for the cell, either causing lysis and exacerbating inflammation, or promoting phagocytosis to remove debris efficiently.

2. Specific methods

2.1. Isolation of apoptotic blebs from culture supernatant

Apoptosis was induced in IMR-32 with CPT, but serum-free RPMI was used instead of RPMI supplemented with 5% FCS, to reduce non-specific binding during Western blotting carried out on these samples. Cells (5 x 10^6) and culture supernatant (18ml) were harvested and each centrifuged at 300g for 5 minutes. To the cell pellet, 200µl lysis buffer was added, and kept on ice for 30 minutes. The lysate was then spun at 16,000g at 4°C in a microfuge for 15 minutes, and the pellet discarded. The lysate supernatant was frozen at –20°C until analysis.

The culture supernatant was spun at 500g for 10 minutes at 4°C and the pellet discarded. The supernatant was concentrated 10 times in an Amicon concentration system with a 10kDa cut-off filter at 4°C. The cell-free supernatant was then spun at 100,000g for 1 hour at 4°C in a Beckman TL-100 ultracentrifuge using a TLA 100.3 rotor. The supernatant was collected, and the pellet dissolved in 1x SDS-PAGE loading buffer, boiled, and then frozen at –20°C until analysis. Proteins remaining in the supernatant were precipitated with 10% TCA for 30 minutes on ice. The samples were then spun at 16,000g at 4°C for 10 minutes, and the pellet was washed twice in acetone and spun at 16,000g for 5 minutes at 4°C before discarding the supernatant and vacuum drying the pellet. The pellet was then dissolved in 1x SDS-PAGE loading buffer, boiled and frozen at –20°C until analysis.

2.2. Phagocytosis assay

2.2.1. Phagocytosis of zymosan-Alexa488 bioparticles

Macrophages were isolated and differentiated as described in Materials and Methods. Macrophages

were released from the plate by replacing the medium with PBS + 10mM EDTA for 30 minutes, and then gently scraping the cell monolayer. Cells were washed twice in serum-free RPMI and labelled with the red fluorescent membrane label PKH26 kit (Sigma) according to the manufacturer's instructions. Cells ($2x10^5$) were plated out into 6 well plates containing poly-D-lysine coated coverslips, and allowed to adhere in RPMI with 5% HI-NHS for 2 days at 37°C, 5% $C0_2$. Macrophages were washed twice in serum-free RPMI and then incubated for 1 hour with zymosan-Alex-488 bioparticles ($2x10^6$ per well) that were either not opsonised (1 hour in RPMI + 5% FCS, then washed twice in serum-free RPMI) or opsonised with C (1 hour in RPMI + 5% NHS, then washed twice in serum-free RPMI). The macrophage monolayer was then washed twice with PBS, dried, fixed in acetone for 30 seconds, mounted on slides using Vectashield (Vector Laboratories, Peterborough, UK) and viewed using a fluorescence microscope (Leica Microsystems, Milton Keynes, UK) or analysed using a Compucyte laser scanning cytometer (LSC; Olympus, London, UK).

2.2.2. Phagocytosis of IMR-32 cells

IMR-32 were labelled with the red fluorescent membrane label PKH26 kit (Sigma), according to the manufacturer's instructions, and allowed to adhere overnight. Apoptosis was induced in IMR-32 using CPT or UV as described in Chapter 2. In some experiments, established apoptotic cells (floating or loosely adherent) were separated from adherent surviving and early apoptotic cells by striking the plate and separately harvesting the two populations. Macrophages were isolated and differentiated as described in Materials and Methods. Macrophages were washed twice in serum-free RPMI and labelled with 2μM calcein.AM in serum-free medium for 45 minutes at 37°C. Cells were washed twice in medium containing 5% FCS, and then twice in serum-free medium. Labelled apoptotic or control IMR-32 cells ($5x10^5$) were then plated onto the near-confluent adherent macrophage monolayer with or without C8d or HI-C8d. After 2 hours, remaining IMR-32 were

washed from the macrophage monolayer using three washes of PBS. Coverslips were then dried, fixed in acetone for 30 seconds, stained with DAPI, and mounted using Vectashield. Samples were analysed using a Leica fluorescence microscope and on a LSC.

3. Results

3.1. CPT, ceramide and UV irradiation induce apoptosis in IMR-32 neuroblastoma cells

The human neuroblastoma cell line IMR-32 was selected for this study, as it closely resembles primary human neurons in the mechanism of C activation, and in its CReg profile (Gasque, Thomas et al. 1996; Singhrao, Neal et al. 2000). These cells possess CD46 and CD59, but are deficient in CD55 even in response to several pro-inflammatory stimuli (van Beek, van Meurs et al. 2005). Apoptosis was induced in IMR32 neuroblastoma cells using three methods: addition of 1μM CPT, a topoisomerase I inhibitor; addition of a combination of 60μM each of C2 and C6 ceramide; or UV irradiation. Figure 3.1A shows that each protocol induced apoptosis as assessed using the apoptosis markers caspACE (FITC-VAD-fmk), and annexin-V-FITC. Early apoptotic cells were defined as stained for annexin-V-FITC and caspACE, but not for PI, indicating membrane integrity was still intact. Cells in late apoptosis stained positively for caspACE, annexin-V-FITC and PI. Late apoptotic cells in the CPT treated group had lower caspACE fluorescence than early apoptotic cells but higher than viable cells, compared to UV treated cells, where late apoptotic cells had the same caspACE fluorescence as early apoptotic cells. The basis for this difference is not clear. In control cells at 0h a small percentage of cells were positive for PI but caspACE negative, indicating that they were necrotic; most likely due to an artefact of handling during the staining procedure. Using these criteria, at 24 hours CPT treated IMR-32 were 23-25% in early apoptosis and 25% in late apoptosis, ceramide treated cells were 5-10% in early apoptosis, and 50% in late apoptosis, and UV treated cells were 60-65% in early apoptosis and 15-20% in late apoptosis. Apoptosis was confirmed in UV treated cells by Western blotting for PARP cleavage (fig 3.1B). PARP is cleaved by caspases into two fragments of 112kDa and 28kDa, demonstrated in figure 3.1B by the use of mAb that recognise the different fragments. Nuclear morphology was visualised by DAPI staining in both CPT treated and UV

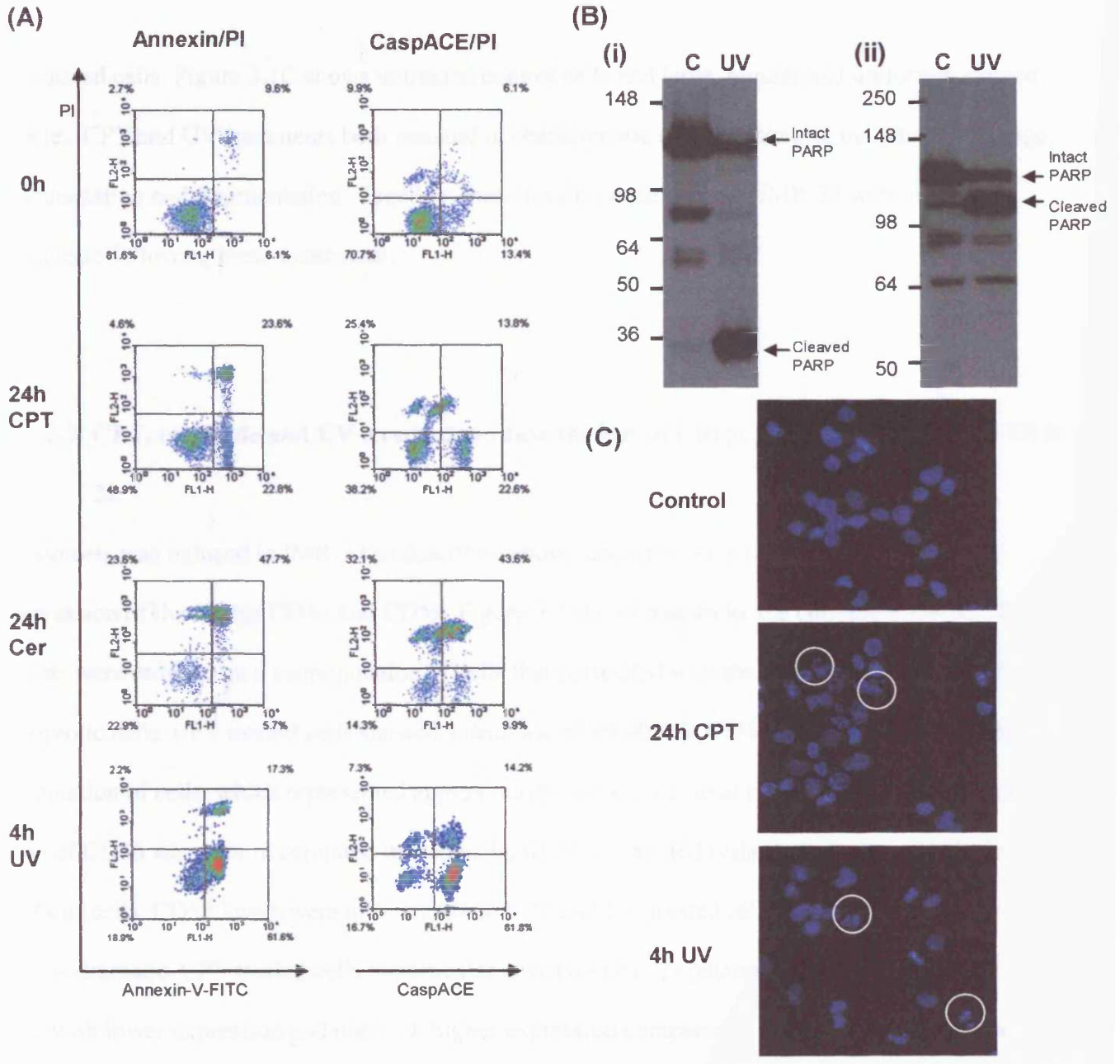


Figure 3.1: Induction of apoptosis in CPT treated, ceramide treated and UV irradiated IMR-32. **(A)** Annexin-V-FITC, caspACE, and PI staining and flow cytometry. Viable cells: Annexin-/caspACE-/PI-. Early apoptotic cells: Annexin+/caspACE+/PI-. Late apoptotic cells: Annexin+/caspACE+/PI+. All induction agents induced apoptosis in IMR-32, but with different kinetics. **(B)** PARP cleavage following UV treatment of IMR-32**.** To confirm that IMR-32 treated with UV irradiation were undergoing apoptosis, PARP cleavage was assessed by Western blot using antibodies that recognise different parts of the cleaved molecule. UV treatment shows a reduction in intact PARP, and in (i) there is an increase in the small cleaved fragment (28kDa) and in (ii) an increase in the large cleaved fragment (112kDa). C=control cells. **(C)** DAPI staining for nuclear morphology. CPT and UV treated cells had smaller, fragmented nuclei compared to control. White circles indicate examples of apoptotic nuclei.

irradiated cells. Figure 3.1C shows untreated control cells had large, regular and uniformly stained nuclei. CPT and UV treatments both resulted in characteristic nuclear changes, including shrinkage, condensation and fragmentation. Together, these results confirmed that IMR-32 were indeed apoptotic following these treatments.

3.2. CPT, ceramide and UV irradiation cause the loss of CRegs CD46 and CD59 from IMR-32

Apoptosis was induced in IMR-32 as described above, and cells were analysed for cell surface expression of the CRegs CD46 and CD59. Figure 3.2 shows that under the conditions tested CD46 levels were reduced on a subpopulation of cells that correlated with the observed percentage of apoptotic cells. CPT treated cells showed a decrease of 80-90% in CD46 expression on the apoptotic population of cells, which represented approximately 48% of the total population. A similar degree of loss of CD46 was seen in ceramide treated cells, while UV treated cells showed a loss of CD46 on 74% of cells. CD59 levels were also altered on CPT and UV treated cells, although the change was not as dramatic. CPT treated cells in particular gave two clear populations of CD59 expressing cells, one with lower expression and one with higher expression compared to controls. The reduction in CD59 on the lower expressing population of CPT treated cells was 25-30%, and this represented approximately 50% of the total population. CD59 expression fell in the UV treated cells by 30% over the whole population, but little change was seen in ceramide treated cells at this time point.

Time course studies were performed on CPT treated IMR-32 to assess whether the loss of CD46 and CD59 was indeed occurring on cells coincident with apoptosis. CPT was chosen to induce apoptosis, as the stages of apoptosis were clearly defined and were easy to follow in a time course. Figure 3.3 shows the time course through apoptosis using annexin-V-FITC and PI. It was clear from these

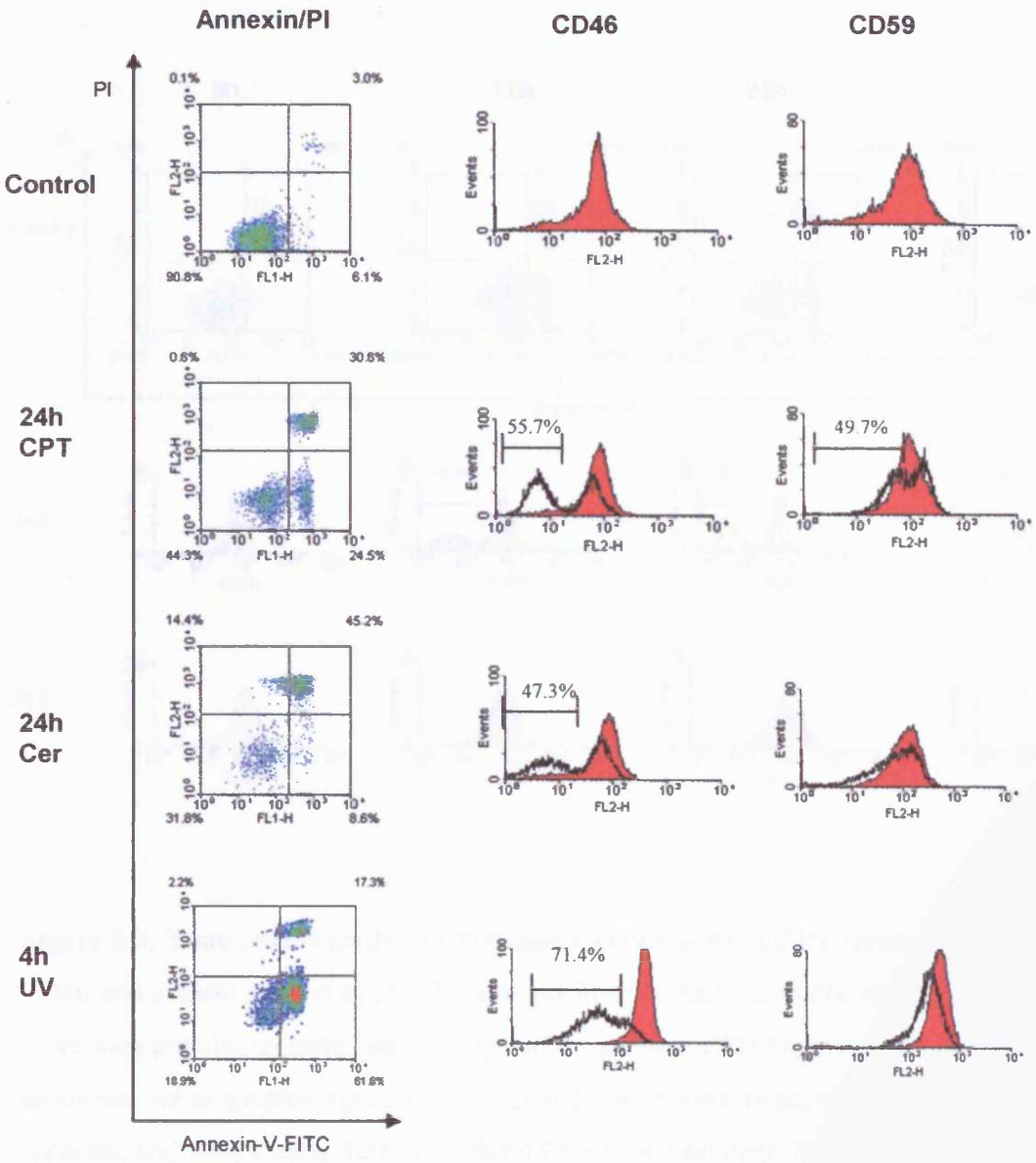


Figure 3.2: Induction of apoptosis and CReg profiles in CPT treated, ceramide treated and UV irradiated IMR-32. All induction agents caused IMR-32 to lose CD46 and CD59. The numbers of cells in the low expressing population were similar to the numbers of apoptotic cells present (early + late apoptotic cells), particularly in relation to CD46. Red filled profiles indicate the control populations at the indicated time point, and the black lines represent the treated populations. Results shown are representative of 3-5 separate experiments.

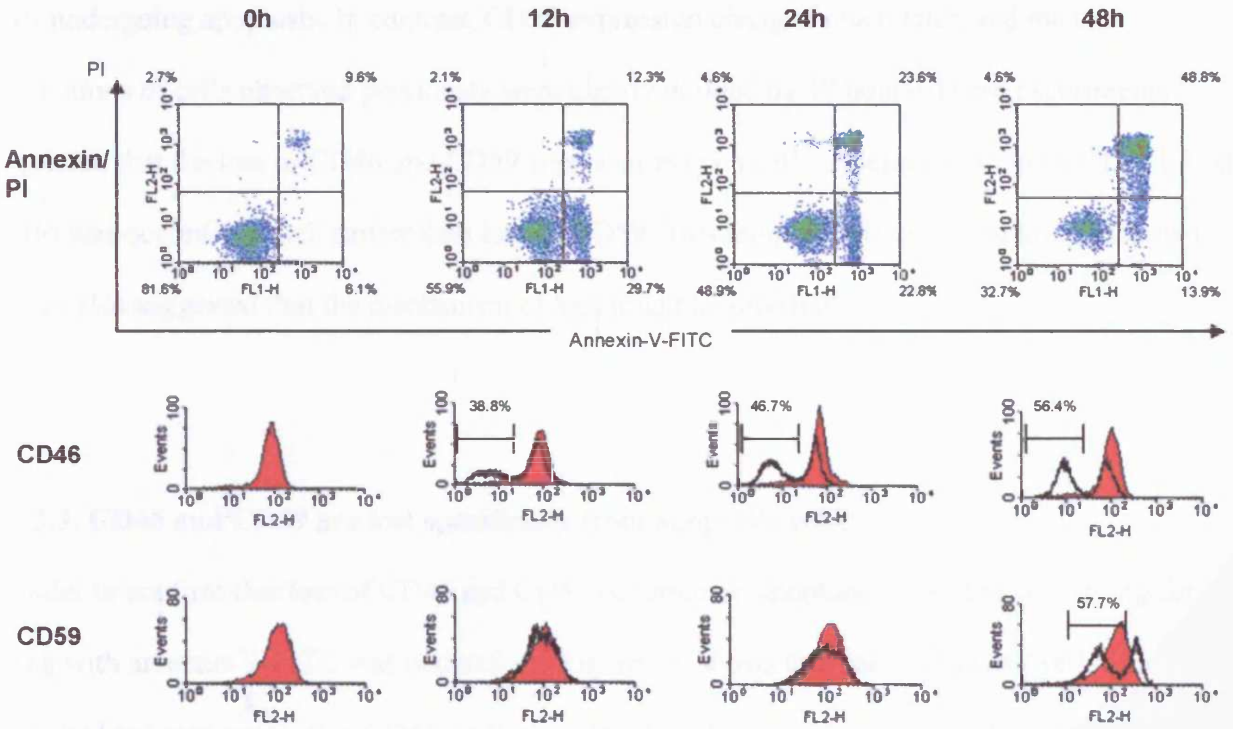


Figure 3.3: Time course study of CD46 and CD59 loss from CPT treated cells. Loss of CD46 was evident as soon as cells became positive for early apoptotic markers, and the low expressing population increased as apoptosis progressed. CD59 loss was not evident early in apoptosis, but as apoptosis progressed high and low expressing populations of cells become apparent, and were clearly definable after 48 hours of treatment. Filled profiles indicate the control populations and black lines represent the CPT treated populations. Results are representative of three experiments

studies that CD46 was lost early during apoptosis, and the extent of loss mirrored the numbers of cells undergoing apoptosis. In contrast, CD59 expression changed much later, and the two populations of cells observed previously were clearly defined by 48 hours. These experiments suggested that the loss of CD46 and CD59 was occurring on cells undergoing apoptosis, and loss of CD46 was occurring much earlier than loss of CD59. This temporal difference in loss of the two CRegs also suggested that the mechanism of loss might be different.

3.3. CD46 and CD59 are lost specifically from apoptotic cells

In order to confirm that loss of CD46 and CD59 occurred on apoptotic cells, double staining for CReg with annexin-V-FITC was undertaken. Figure 3.4 shows that only cells positive for annexin-V-FITC had reduced expression CD46, indicating that the phenomenon is closely linked with the process of apoptosis. Double staining for CD59 and annexin-V-FITC clearly shows the separation into two populations at 24 hours, with the apoptosis positive population expressing lower levels of CD59. It was also noted that 10-15% of the cells in the control population stained with annexin-V-FITC. This most likely represents an artefact caused by handling during the staining procedure, as similar levels were also seen in naïve cells (Figure 3.1A, 0h).

3.4. CD200 is lost from apoptotic IMR-32

Since apoptotic IMR-32 lost CRegs during apoptosis, it was hypothesised that other neuronal immune modulators would also be affected. CD200 is a negative modulator of microglia and macrophage activation. Loss of CD200 from neuronal cells would therefore result in activation of these cells. Figure 3.5 demonstrates that CD200 is expressed by control IMR-32. During apoptosis induced by either CPT or UV, apoptotic cells lost CD200 (42-50%; $p<0.01$) from their cell surface. CD200 levels

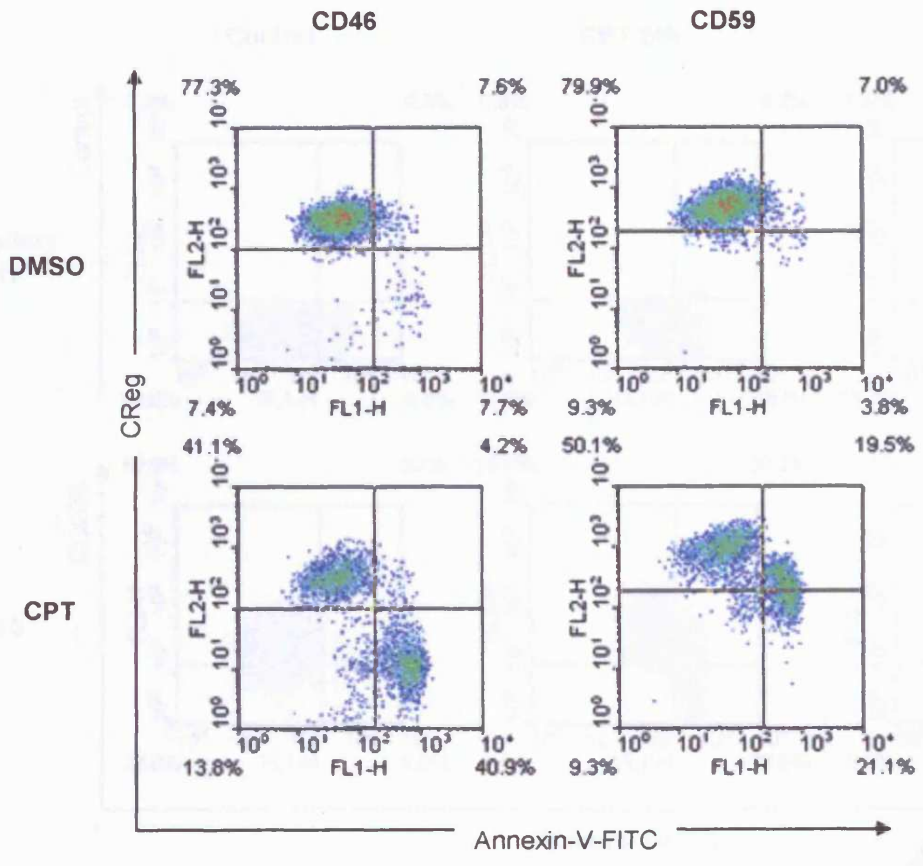


Figure 3.4: Double staining apoptotic IMR-32 for CReg and apoptosis markers. Distinct populations after 24h of CPT treatment confirm that only cells positive for apoptosis markers have reduced levels of CD46 and CD59. Results are representative of three experiments.

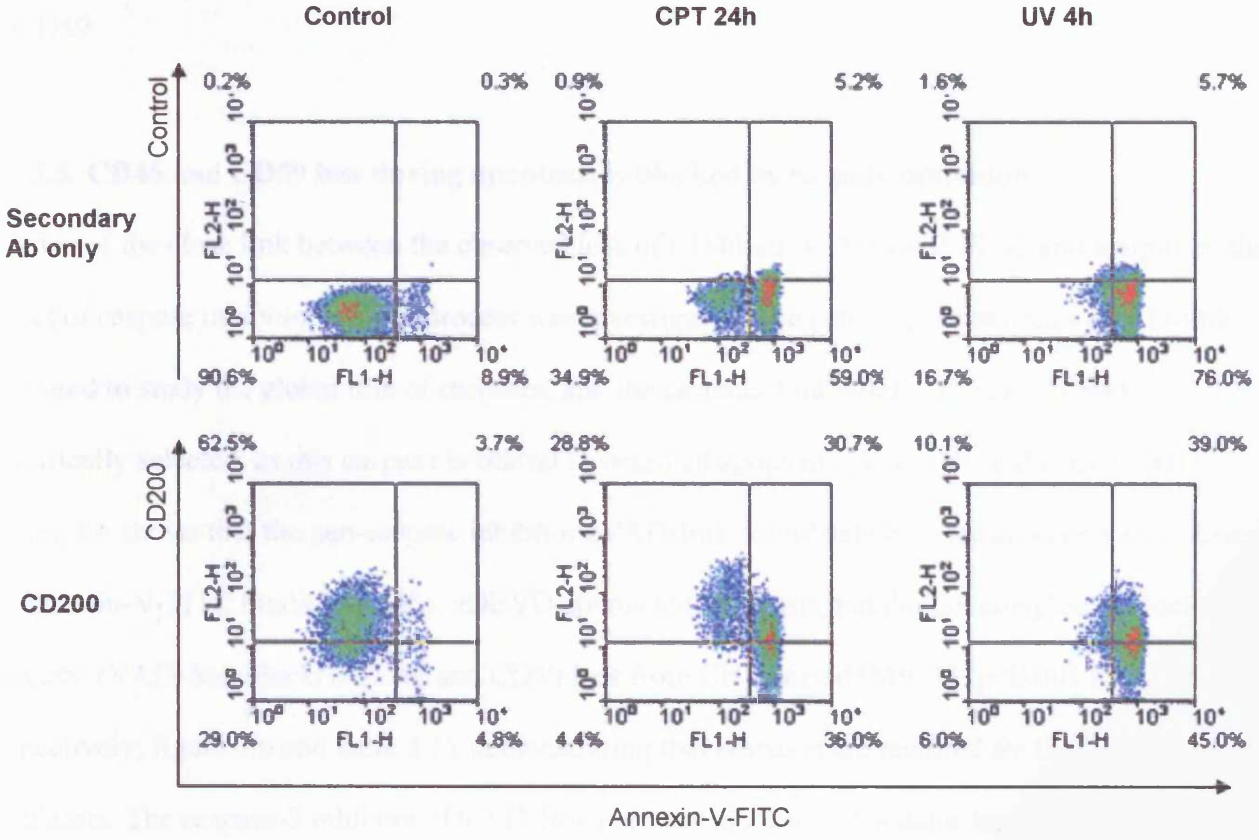


Figure 3.5: Double staining apoptotic IMR-32 for CD200 and apoptosis markers. Distinct populations after 24h of CPT or 4h UV treatment confirm that cells positive for apoptosis markers have reduced levels of CD200. Surviving cells in the CPT treated population show increased CD200 expression. Results are representative of three experiments.

increased on the surviving population of CPT treated cells by two and a half fold, in a similar fashion to CD59.

3.5. CD46 and CD59 loss during apoptosis is blocked by caspase inhibition

In view of the close link between the observed loss of CD46 and CD59 on IMR-32 and apoptosis, the effect of caspase inhibition on this process was investigated. The pan-caspase inhibitor zVAD-fmk was used to study the global role of caspases, and the caspase-3 inhibitor zDEVD-fmk was specifically selected, as this caspase is central to neuronal apoptosis (Yakovlev and Faden 2001). Figure 3.6 shows that the pan-caspase inhibitor zVAD-fmk completely blocked apoptosis as assessed by annexin-V-FITC binding to cells. zDEVD attenuated apoptosis, but did not completely block the process. zVAD-fmk blocked CD46 and CD59 loss from CPT treated IMR-32 ($p<0.001$ and $p<0.001$ respectively; figure 3.6 and table 3.1), demonstrating that caspases are required for the loss of C regulators. The caspase-3 inhibitor zDEVD-fmk partially blocked C regulator loss (CD46 $p<0.001$, CD59 $p<0.001$; figure 3.6 and table 3.1), indicating that although caspase 3 is important in the mechanism of loss, other caspases are also involved. It was possible that some of the changes observed might be due to cell shrinkage, well reported in apoptosis. This issue was addressed by measuring forward scatter profiles for each population. Similar changes in cell size were seen with CPT treatment in the presence or absence of caspase inhibitors (figure 3.6), suggesting that some aspects of the apoptotic process are unaffected by caspase inhibition. The data also confirm that the apparent loss of CReg was not due to an artefact of cell size change as CReg loss is blocked by the caspase inhibitors but cell size change is unaltered.

3.6. CD46 and CD59 are shed into the culture supernatant during apoptosis

The fate of CD46 and CD59 lost from the surface of apoptotic cells was then investigated. Apoptosis was induced as described previously with CPT, but in serum-free medium to avoid serum-related

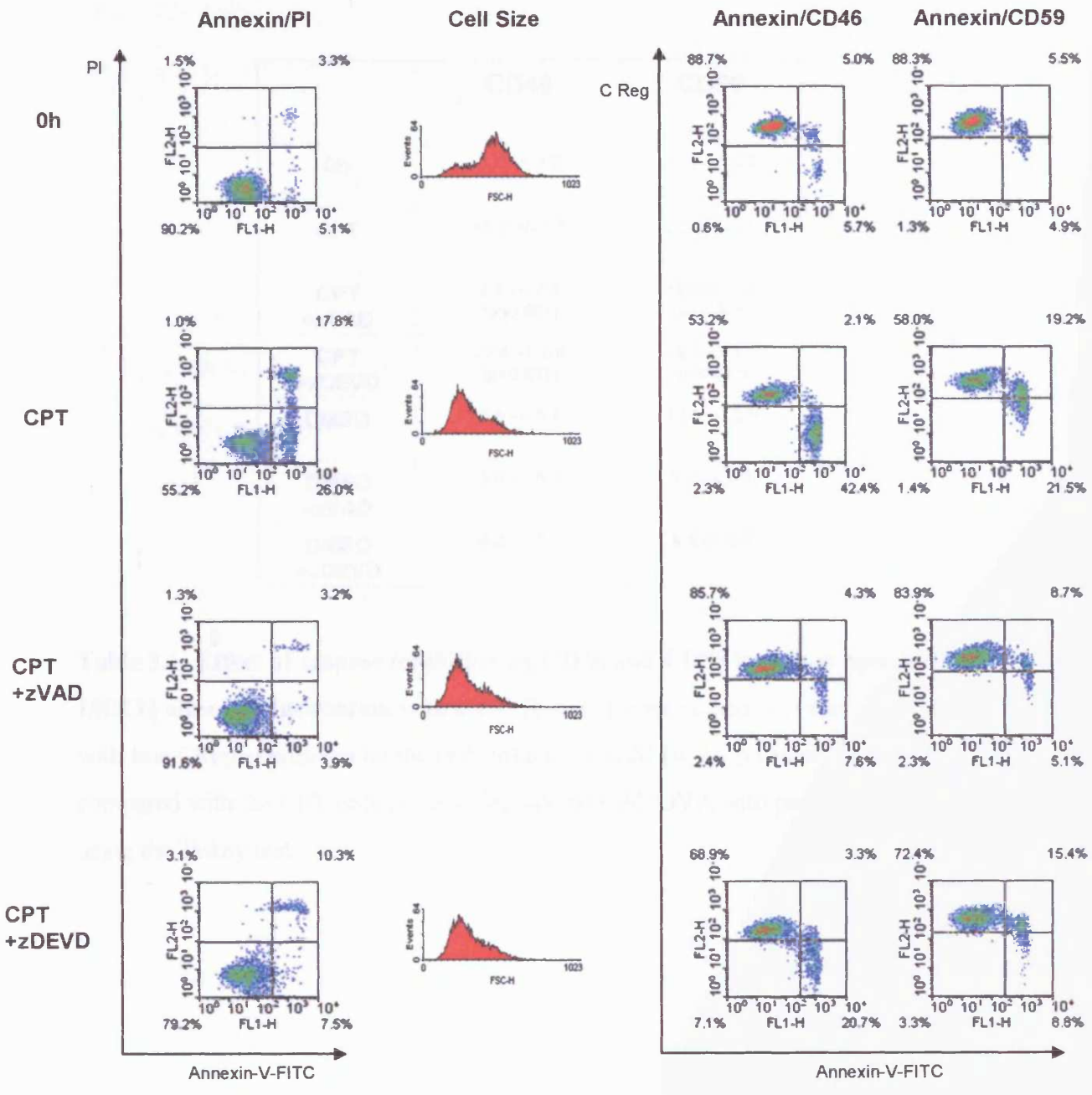


Figure 3.6: Effect of caspase inhibition on CD46 and CD59 loss from apoptotic IMR32 after 24h incubation with CPT. Flow cytometry demonstrated zVAD-fmk and zDEVD-fmk completely and partially, respectively, inhibited apoptosis as assessed by annexin-V-FITC binding, and CReg loss, but not the decrease in cell size. The pan-caspase inhibitor zVAD-fmk abolished loss of CReg, and the caspase 3/7-inhibitor zDEVD-fmk partially inhibited loss. Results representative of three experiments.

	CD46	CD59
0h	8.7 +/- 0.2	11.5 +/- 0.2
CPT	45.0 +/- 1.7	29.8 +/- 1.3
CPT +zVAD	9.4 +/- 0.3 (p<0.001)	13.1 +/- 0.6 (p<0.001)
CPT +zDEVD	22.4 +/- 0.6 (p<0.001)	16.0 +/- 1.5 (p<0.001)
DMSO	7.5 +/- 0.1	10.9 +/- 0.3
DMSO +zVAD	5.9 +/- 0.2	9.2 +/- 0.4
DMSO +zDEVD	6.2 +/- 0.1	9.6 +/- 0.7

Table 3.1: Effect of caspase inhibition on CD46 and CD59 loss from apoptotic IMR32 after 24h incubation with CPT. Results are expressed as percent of cells with low CReg expression on the cell surface +/- SEM (n=3). p values are all compared with the CPT only group using one-way ANOVA with post-hoc analysis using the Tukey test.

background in the Western blots. In the absence of serum, apoptosis was faster and the proportion of cells undergoing apoptosis after 16 hours was similar to that after 24 hours when serum was present (figure 3.7). The supernatant and cell layer were collected, and after TCA precipitation of the supernatant the samples were subject to SDS-PAGE, transferred to nitrocellulose membranes, and immunoblotted. Figure 3.8A shows that CPT treated cells had lost both forms of CD46. CD46 appeared in the culture supernatant from CPT treated cells but not controls. The supernatant contained two CD46 bands; the upper band had a much stronger signal than the lower band on blotting and is more readily seen in figure 3.8B. These bands were of Mr 47 and 51kDa approximately, some 5-8kDa lighter than the respective upper and lower bands present in the cell lysate. This suggested that CD46 was lost preferentially from the cell surface, and that the likely mechanism of shedding was by enzymatic cleavage. CD59, in contrast, was still present at high levels in the lysate from the CPT treated cells, but was also found in abundance in the supernatant from these cells. Cells treated with DMSO as control did not lose CD46 or CD59 from the lysate and no CD46 or CD59 was detected in the supernatant at 16 hours (figure 3.8A).

3.7. CD59 is lost on apoptotic blebs and in a soluble form, and CD46 is shed in a soluble form only

In order to further investigate the mechanism of shedding of CD46 and CD59, an ultracentrifugation step was performed (100,000g for 1 hour) on the cell-free supernatant from CPT treated cells, to separate out proteins in the particulate (apoptotic bleb) fraction from those in the soluble fraction. The pellet was resuspended in SDS-PAGE loading buffer; the supernatant was concentrated, and the proteins TCA precipitated. Western blotting was performed, and Figure 3.8B shows a time course study on the supernatant from CPT treated cells. Both CD46 and CD59 were detected in the supernatant from 4 hours, demonstrating that the loss of CReg was a relatively early event. Both

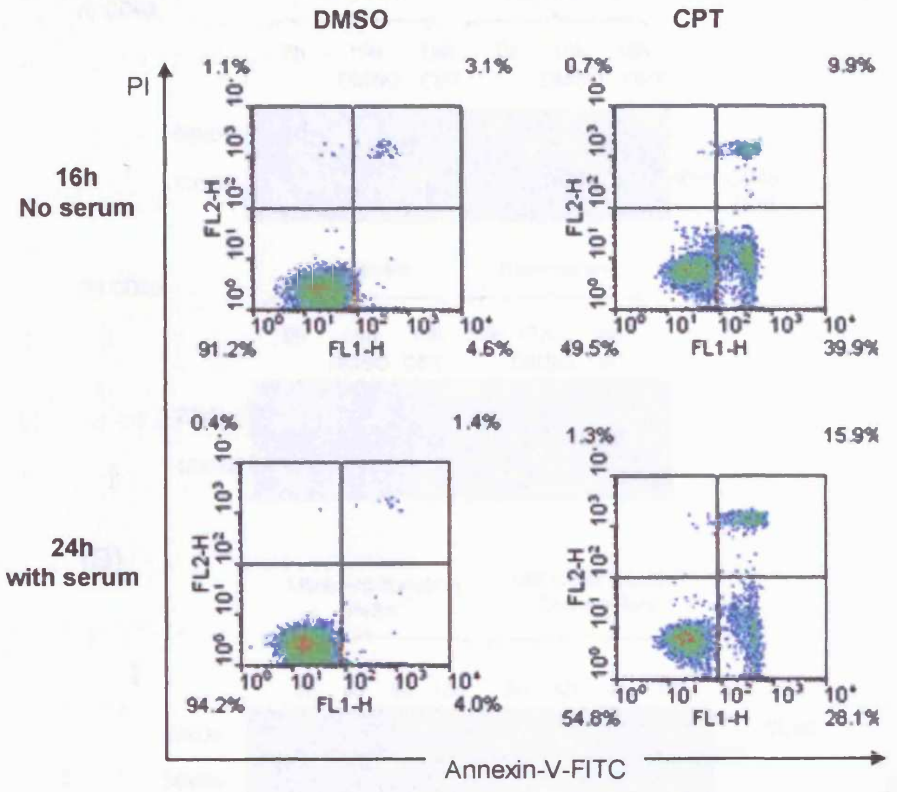


Figure 3.7: Effect of serum on progress through CPT-induced apoptosis. Apoptosis was induced with CPT in IMR-32 in the presence or absence of serum. Serum inhibited the rate of progression of apoptosis, and at 24h numbers of apoptotic cells in the serum group was similar to the numbers of apoptotic cells induced without serum.

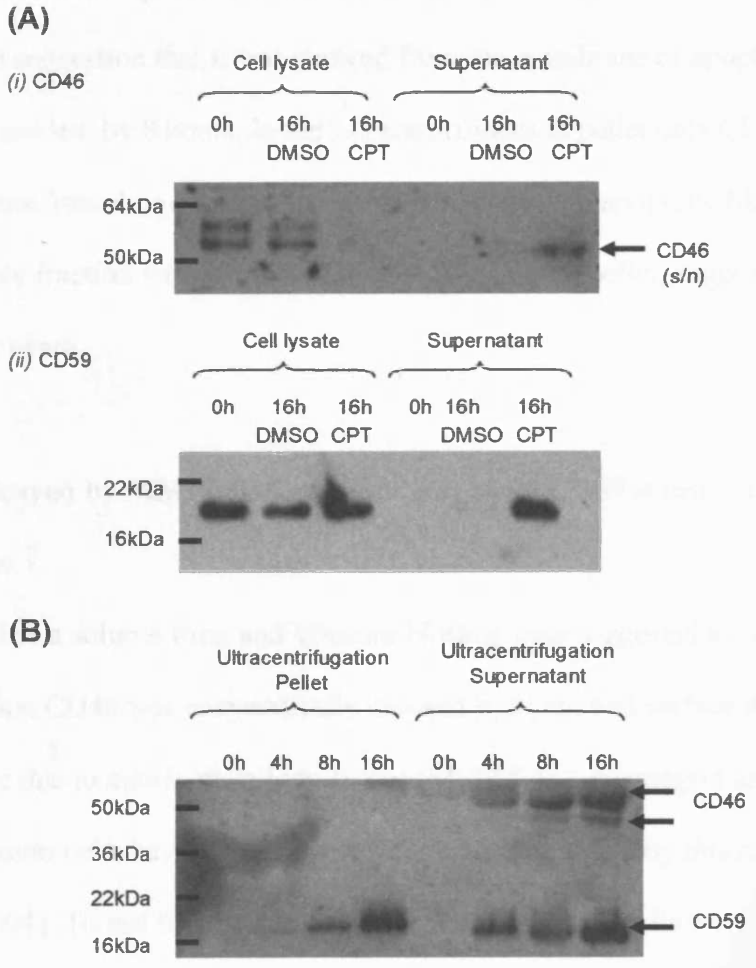


Figure 3.8: Western blotting of CD46 and CD59 in cell lysates and culture supernatant. **(A)** CD59 and CD46 were present in the culture supernatant of CPT treated cells only. CD46 was present as a doublet in the lysate from untreated (0h) and DMSO treated cells, but not seen in the lysate from CPT treated cells. CD59 was present in CPT treated cells at a level equivalent to controls. Cell lysates loaded with 20μg protein per lane. **(B)** Time course of CReg shedding into the supernatant and partitioning by ultracentrifugation at 100,000g. CD59 was present in the ultracentrifugation pellet and also in the soluble fraction of the supernatant from 4h post-exposure to CPT, with increasing amounts present as the time of exposure increased. CD46 was present in the supernatant fraction in increasing amounts from 4 hours, and a second lower Mr form (approx 2kDa lighter) was detectable from 8 hours

proteins accumulated in the supernatant to 16 hours. The presence of CD46 only in the supernatant further supports the suggestion that it was cleaved from the membrane of apoptotic cells, and the two bands were clearly evident by 8 hours. In the ultracentrifugation pellet only CD59 was detected, present in increasing amounts from 4 hours, indicating that it was shed on apoptotic blebs. CD59 was also present in the soluble fraction with Mr 2-3kDa lighter than in the pellet, suggesting that it may also undergo a cleavage event.

3.8. CD46 is cleaved by MMPs during apoptosis, while CD59 is lost via an MMP independent mechanism

Since CD46 is shed as a soluble form and Western blotting data suggested a reduction in apparent Mr, it was hypothesised that CD46 was enzymatically cleaved from the cell surface during apoptosis, and that this was likely to be due to matrix metalloproteases (MMPs) or a disintegrin and metalloprotease (ADAM), since tumour cells have recently been shown to shed CReg by this mechanism (Hakulinen, Junnikkala et al. 2004). To test this CPT treated and DMSO control cells were incubated with or without the broad-spectrum MMP/ADAM inhibitor GM6001 (50μM). The DMSO carrier (no GM6001) inhibited CPT-induced apoptosis to a small degree (10% fewer apoptotic cells), and GM6001 had no additional effect on the progression of IMR-32 through apoptosis (figure 3.9A). Figure 3.9B shows that CD46 loss from the CPT treated cells was partially inhibited by GM6001 at 24 hours, but GM6001 had no effect on CD59 loss. This confirmed that an MMP/ADAM-mediated cleavage event was taking place for CD46, and demonstrated that CD59 loss occurred via an MMP-independent mechanism. To further define which MMPs were involved in the cleavage of CD46, specific inhibitors against MMP-2, MMP-3, MMP-8 and MMP-9, and also TIMP-3, which is an endogenous inhibitor of ADAMs, were tested. Figure 3.10 shows that inhibition of MMP-3 and MMP-8 were the most potent cleavage inhibitors; partial inhibition was also seen with MMP-9 inhibition. No effect was seen with MMP-2 inhibition or with treatment with TIMP-3, suggesting that ADAMs were not involved. Combined inhibition of MMP-

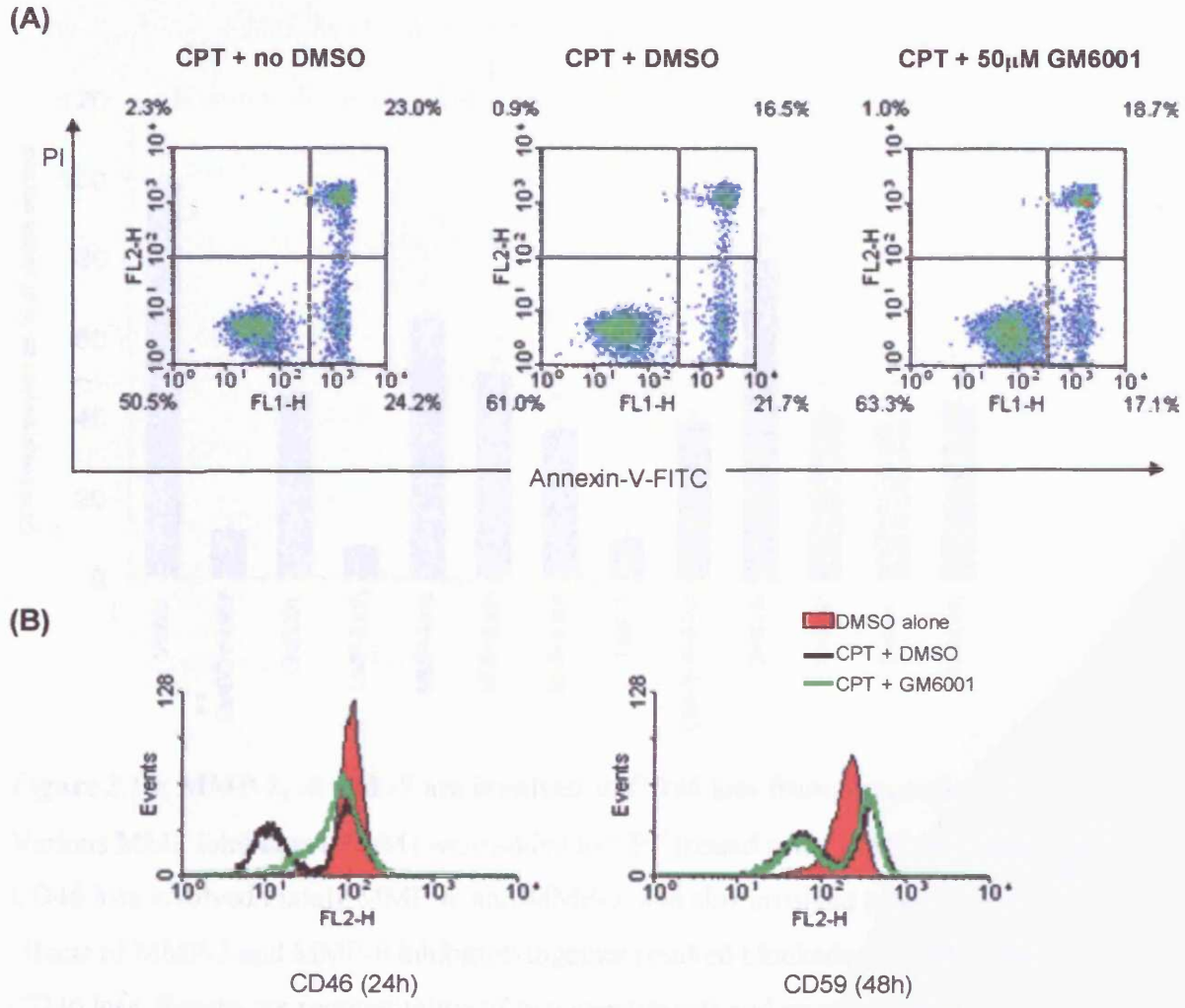


Figure 3.9: Effect of MMP inhibition on CD46 and CD59 loss from apoptotic IMR-32. The MMP inhibitor GM6001 (50μM) was added to CPT treated and DMSO control cells. **(A)** Apoptosis analysis using annexin-V-FITC/PI showed that increased DMSO (carrier control) caused a small inhibition of apoptosis, but GM6001 had no additional effect at the concentrations used. **(B)** CD46 loss was inhibited by GM6001, but there was no effect on CD59 loss at 48 hours. Results are representative of three experiments.

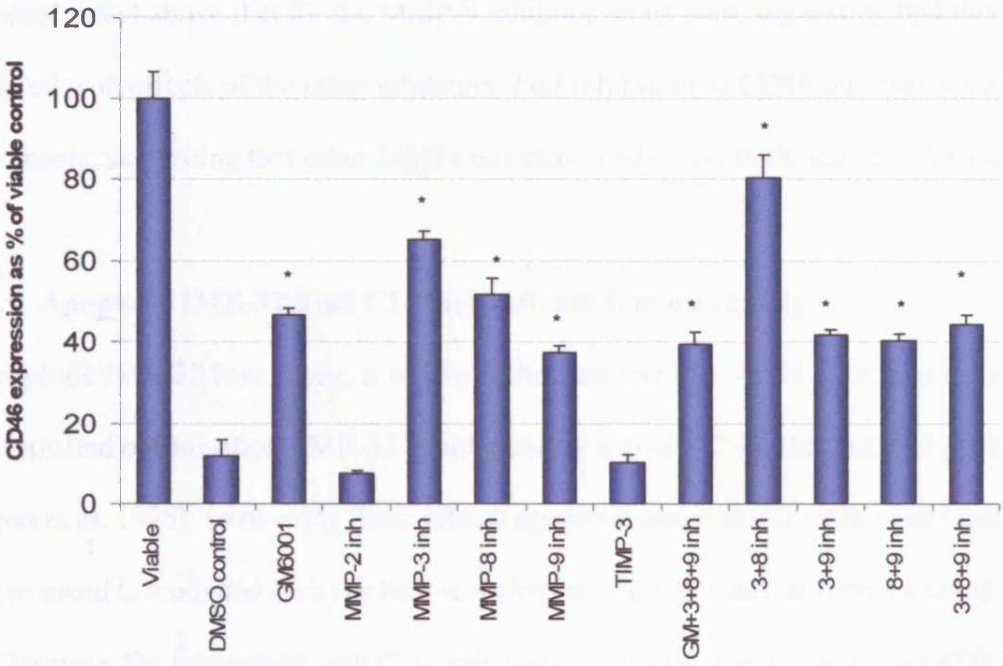


Figure 3.10: MMP-3, -8 and -9 are involved in CD46 loss from apoptotic IMR-32. Various MMP inhibitors (50μM) were added to CPT treated cells. MMP-mediated CD46 loss involved mainly MMP-8, and MMP-3, and also involved MMP-9. The effects of MMP-3 and MMP-8 inhibition together resulted blockade of most of the CD46 loss. Results are representative of two experiments and expressed as mean +/- SD. Significant differences are in comparison with DMSO control. * $p<0.001$.

3 and MMP-8 blocked the majority of CD46 loss, indicating that these MMPs functioned independently. MMP-9 inhibition in combination with other MMP inhibitors did not have any additional effect above that for the MMP-9 inhibitor on its own, suggesting that this inhibitor interfered with effects of the other inhibitors. Full inhibition of CD46 loss was not achieved in these experiments, suggesting that other MMPs not examined in the study may also be involved.

3.9. Apoptotic IMR-32 bind C1q and activate C more readily

As apoptotic IMR-32 lose CReg, it was hypothesised that this would alter their susceptibility to C activation and opsonisation. IMR-32 spontaneously activate C via the classical pathway (Gasque, Thomas et al. 1996), so to study these effects apoptotic and controls cells were incubated with 20% C8d (to avoid C mediated lysis) or heat-inactivated C8d (HI-C8d), and then stained with antibodies to C3b. To study the interaction with C1q cells were incubated directly with C1q-FITC, since in preliminary experiments detection of C1q with antibodies following exposure to serum resulted in very high background and were difficult to interpret. Double staining was performed using annexin-V-FITC, or PI. Figure 3.11 shows that IMR-32 bound C1q in both control and CPT treated populations, and that BSA-FITC did not bind to either population. Some control cells bound high levels of C1q and all were strongly PI positive, likely representing cells dying during the experimental procedure. In CPT treated cells, the percentage of C1q-bright cells was higher (12% vs 17%) and all were PI positive, suggesting they were late apoptotic cells. This suggested that early apoptotic cells did not bind more C1q than controls, and that increased C1q binding only occurred on a subpopulation of PI+ cells, most likely those in late apoptosis or necrosis.

Figure 3.12A demonstrates that 10-fold more C3b was deposited on annexin-V-FITC positive cells in the CPT treated population compared to control cells, indicating that apoptotic cells activated C more readily. Double staining cells for CD46 and C3b supported this observation (figure 3.12B). The

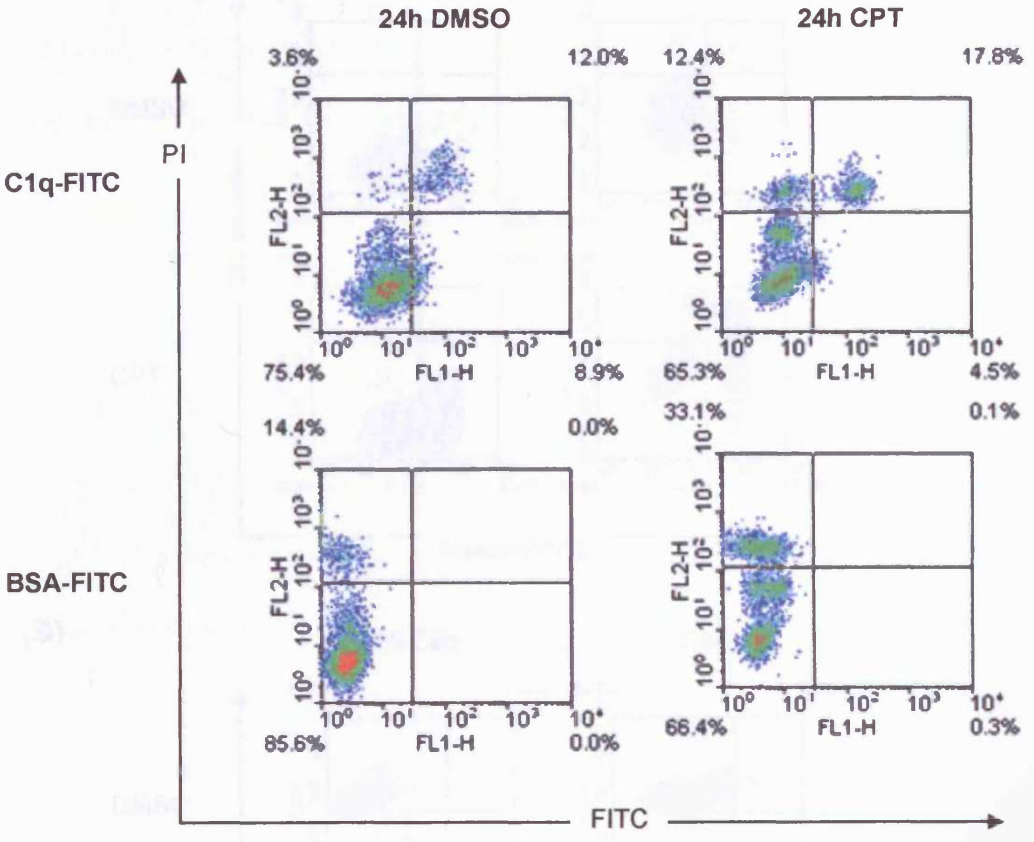


Figure 3.11: C1q binding on apoptotic cells. C1q binding was increased only on a subset of cells with high PI fluorescence; CPT treatment increased this population by 5-6% over control. C1q bound to viable control cells, and no increase was seen on viable CPT treated cells. BSA-FITC did not bind control or CPT treated cells.

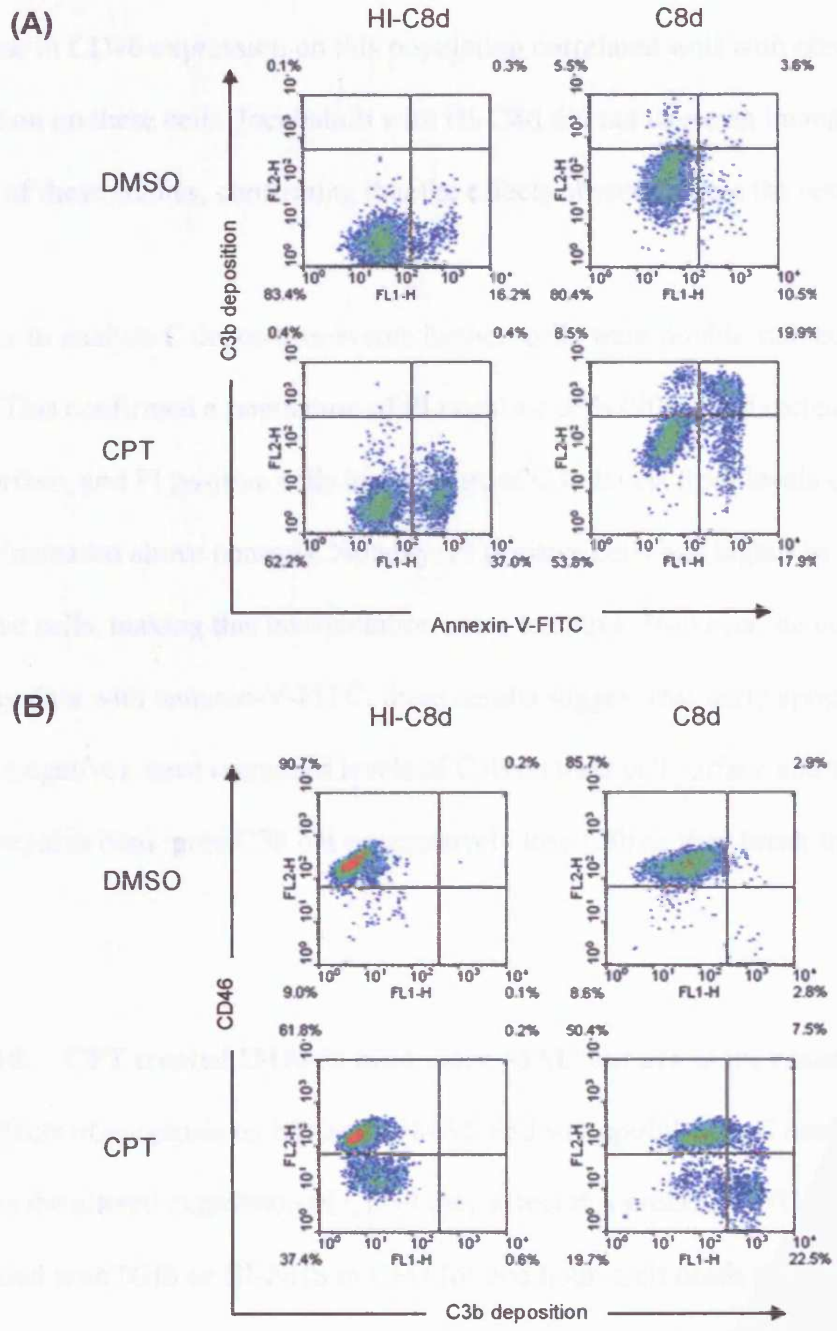


Figure 3.12: C activation on apoptotic cells. (A) C3b binding was markedly increased on CPT treated and annexin-V-FITC positive cells. The annexin-V-FITC negative population in the CPT treated population had slightly increased C3b binding compared to the control population. **(B)** Deposition of C3b was highest on cells that had lost most of their CD46; those in the CD46 high population (CPT treated group) also bound more C3b. HI-C8d controls in these experiments confirmed that deposition was dependent on C activation. Results are representative of three experiments.

decrease in CD46 expression on this population correlated well with observed increase in C activation on these cells. Incubation with HI-C8d did not cause an increase in the deposition of C3b in any of these studies, confirming that the effects observed were the result of C activation.

In order to analyse C deposition events further, cells were double stained for C3b and PI (figure 3.13). This confirmed a population of PI negative cells (20%) had increased levels of C3b on their cell surface, and PI positive cells had a range of C3b levels from levels equivalent to controls to levels increased above controls. Notably, PI positive cells had higher background staining than PI negative cells, making this interpretation more tentative. However, taken together with double staining data with annexin-V-FITC, these results suggest that early apoptotic cells (annexin positive and PI negative), have increased levels of C3b on their cell surface and that late apoptotic cells (PI positive) also bind more C3b but progressively lose C3b as they break up.

3.10. CPT treated IMR-32 bind more MAC but are more resistant to C-mediated lysis

The effects of apoptosis on binding of MAC and susceptibility to C mediated lysis were examined next, as the altered expression of CD59 may affect this process. CPT treated and control cells were incubated with NHS or HI-NHS in CFD for one hour. Cell death was assessed by measurement of LDH in the cell-free supernatant, and staining with an anti-C9 neoepitope mAb (B7) and analysis by flow cytometry was used to assess the extent of MAC deposition on the cell surface. Despite the large increase in C activation observed on apoptotic cells (figure 3.12A), after 12 hours of CPT treatment there was no change in MAC binding (figure 3.14A) or susceptibility to lysis (figure 3.14B). Surprisingly, after 24 hours of CPT treatment, a decreased susceptibility to lysis was evident with 10% serum, despite the increase in MAC binding.

To further analyse this phenomenon, apoptotic cells (floating or loosely adherent) were separated

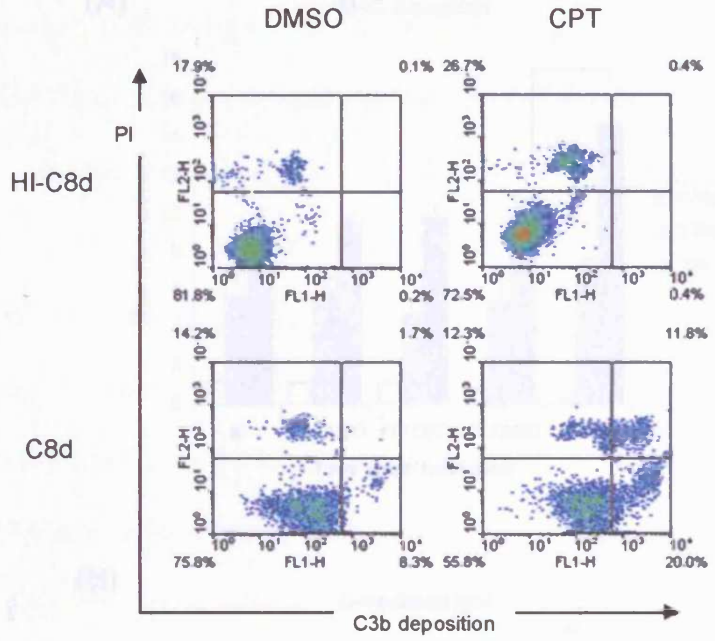


Figure 3.13: C activation on apoptotic cells. C3b binding was markedly increased on CPT treated cells compared to DMSO controls. High C3b binding was seen on both PI- (20%) and PI+ (11.8%) cells. HI-C8d controls showed that the PI+ population had higher background fluorescence compared to PI- cells. Results are representative of two experiments.

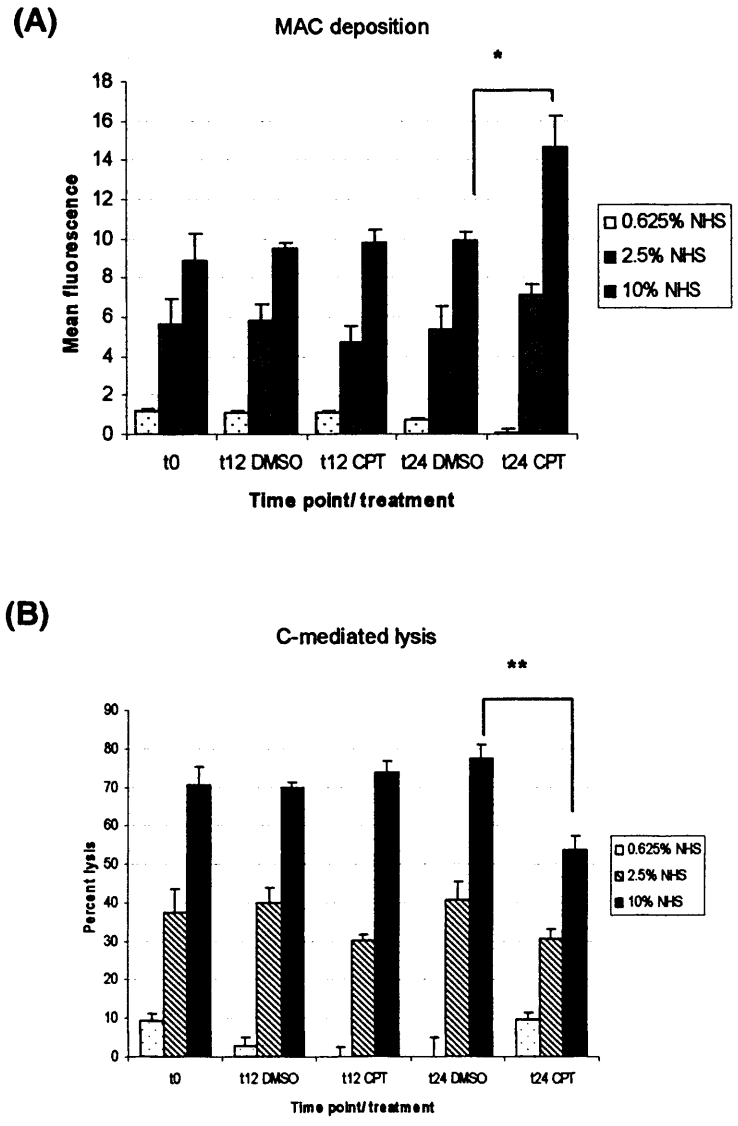


Figure 3.14: MAC binding and susceptibility to lysis in CPT treated IMR-32. CPT treated IMR-32 show increased binding of MAC by flow cytometry after 24h of treatment, but are more resistant to C mediated lysis as detected by LDH release. Results are corrected using subtraction of HI-NHS controls. Results are mean +/- SEM (n=4) from three combined experiments. * $p<0.05$, ** $p<0.01$.

from surviving (adherent) cells and each fraction subjected to a lysis assay. Separation resulted in populations with 90-95% of cells in apoptosis, or 90-95% viable cells (figure 3.15). To achieve equivalence with C-deposition assays, serum was diluted in RPMI and cells were subject to a lysis assay using PI staining to identify dead cells. Figure 3.16A shows that at 12 hours the two populations behaved in a markedly different manner, with apoptotic cells more susceptible to C-mediated killing, and surviving cells more resistant. In the previous assay (figure 3.14B) these effects most likely cancelled out in the mixed population, thus appearing to result in no change in lytic susceptibility. At 24 hours both apoptotic cells and surviving cells were more resistant to killing than controls (figure 3.16B). In keeping with apoptotic cells after 12h CPT, apoptotic cells after 4h UV also showed an increased susceptibility to lysis (figure 3.16C).

3.11. MMP inhibitors block increased C deposition and increased C-mediated lysis of apoptotic IMR-32

Since MMP inhibitors blocked CReg loss from apoptotic IMR-32, it was hypothesised that this would lead to a reduction in C3b deposition on apoptotic cells, and a decrease in C-mediated lysis. UV treated cells were incubated with either 20% C8d or NHS, with HI-C8d or HI-NHS controls, for the C-deposition and lysis assays respectively. Figure 3.17A shows that GM6001 partially blocked CD46 loss from apoptotic IMR-32 in a similar fashion to CPT treated cells. GM6001 also reduced C3b deposition on UV treated cells (figure 3.17B), suggesting that the increase seen following induction of apoptosis was indeed due to CD46 loss. Figure 3.17C shows that inhibition of MMPs with GM6001 also attenuated the increase in lytic susceptibility seen with apoptotic cells, adding further weight to the contention that increased C activation as a result of CD46 loss was at least partially responsible for the increased rate of C-mediated killing of these cells.

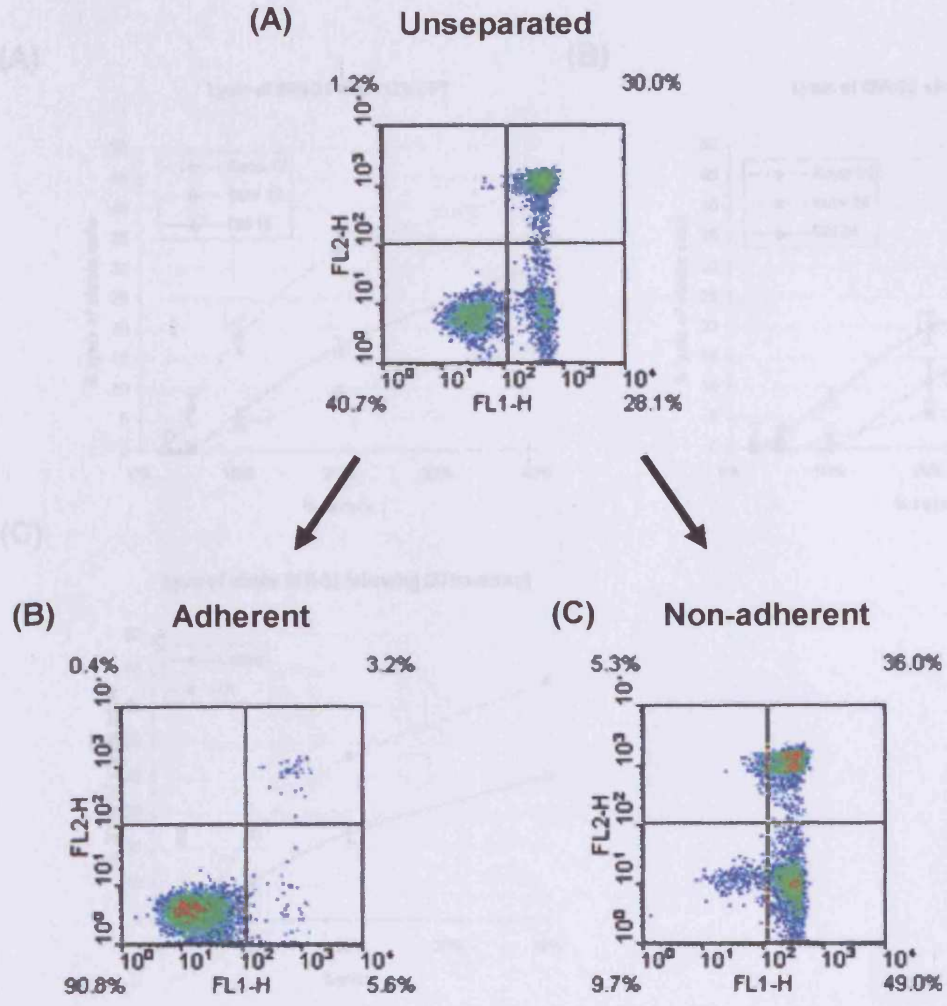
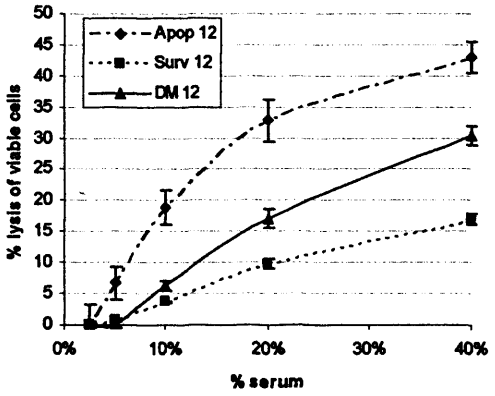
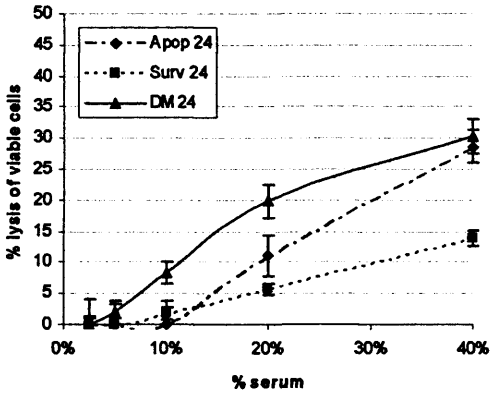


Figure 3.15: Separation of subpopulations of apoptotic and surviving cells following CPT treatment. CPT treated cells exist as two populations: apoptotic cells and viable cells (A). Mechanical separation was used: the flask was struck gently 10 times; those still adherent were 90-95% viable (B). Cells floating in the medium were 90-95% apoptotic (C).

(A)



(B)



(C)

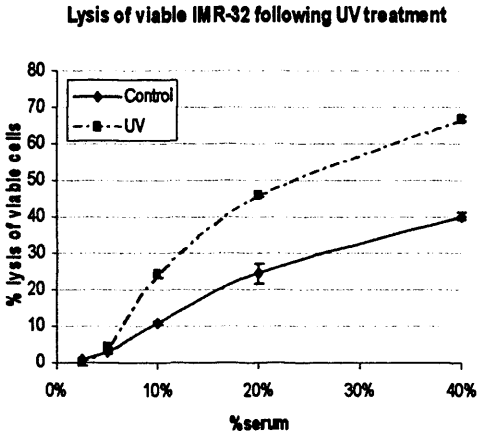


Figure 3.16: Susceptibility to C-mediated lysis in apoptotic IMR-32 cells and cells surviving CPT treatment. CPT treated cells were split into apoptotic (non-adherent) and surviving (adherent) fractions after 12h (A) and 24h (B) and subject to C-mediated lysis using serum in RPMI. Viable (PI-) apoptotic cells were more susceptible to C-mediated lysis than controls at 12h, but more resistant at 24 hours. At both time points surviving cells were more resistant to lysis. UV treated cells were subject to C-mediated lysis (C), and were also found to be more susceptible to killing. Results are combined from three experiments performed in duplicate, and are expressed as mean +/- SEM.

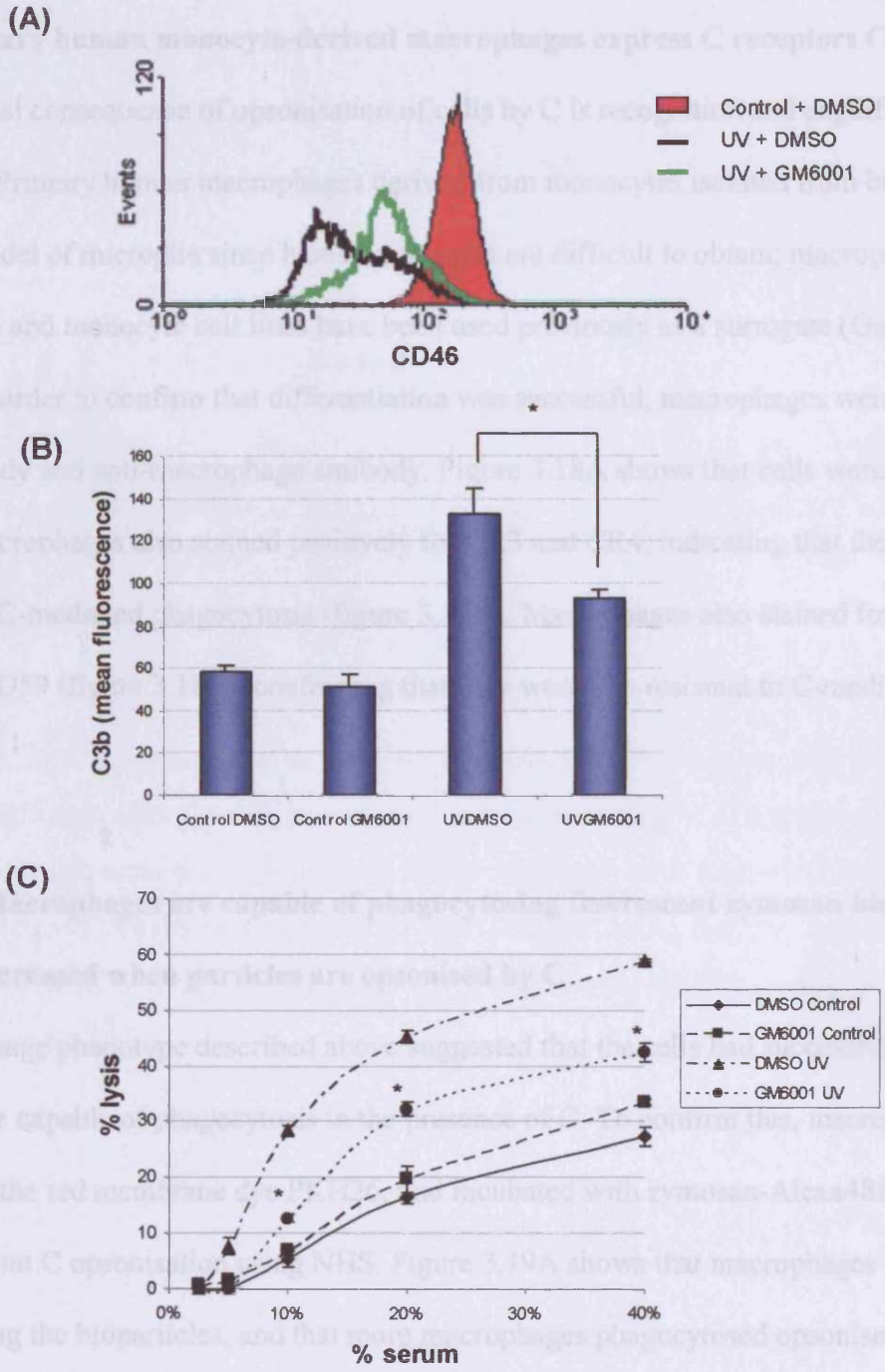


Figure 3.17: Effect of MMP inhibition on C activation and C-mediated lysis in UV treated IMR-32. Cells were incubated with GM6001 (50μM) or DMSO (carrier control) and UV irradiated for 4h. Cells were harvested and C3b deposition assessed or susceptibility to C-mediated lysis assayed. **(A)** CD46 loss from UV irradiated IMR-32 was partially blocked by the inhibition of MMPs by GM6001. **(B)** Increased C3b deposition on UV irradiated cells was attenuated by GM6001. **(C)** Increase in C-mediated lysis of UV irradiated IMR-32 was blocked by MMP-inhibition. * $p < 0.01$, compared to DMSO UV.

3.12. Primary human monocyte-derived macrophages express C receptors CR3 and CR4

The functional consequence of opsonisation of cells by C is recognition and engulfment by phagocytes. Primary human macrophages derived from monocytes isolated from buffy coat were used as a model of microglia since human microglia are difficult to obtain; macrophages share the same lineage and monocyte cell lines have been used previously as a surrogate (Gasque, Fontaine et al. 1995). In order to confirm that differentiation was successful, macrophages were stained with CD14 antibody and anti-macrophage antibody. Figure 3.18A shows that cells were positive for both markers. Macrophages also stained positively for CR3 and CR4, indicating that they had the receptors required for C-mediated phagocytosis (figure 3.18A). Macrophages also stained for CRegs CD55, CD46 and CD59 (figure 3.18B), confirming that they would be resistant to C-mediated attack during the assay.

3.13. Macrophages are capable of phagocytosing fluorescent zymosan bioparticles and this is increased when particles are opsonised by C

The macrophage phenotype described above suggested that the cells had successfully differentiated, and would be capable of phagocytosis in the presence of C. To confirm this, macrophages were stained with the red membrane dye PKH26, and incubated with zymosan-Alexa488 bioparticles either with or without C opsonisation using NHS. Figure 3.19A shows that macrophages were capable of phagocytosing the bioparticles, and that more macrophages phagocytosed opsonised bioparticles. It was also noted that more bioparticles were phagocytosed per macrophage in the presence of C. Phagocytosis was quantified using the LSC (figure 3.19B), and showed that 13-14% of macrophages phagocytosed non-opsonised bioparticles, and 85-86% phagocytosed opsonised bioparticles, confirming the fluorescence microscopy data.

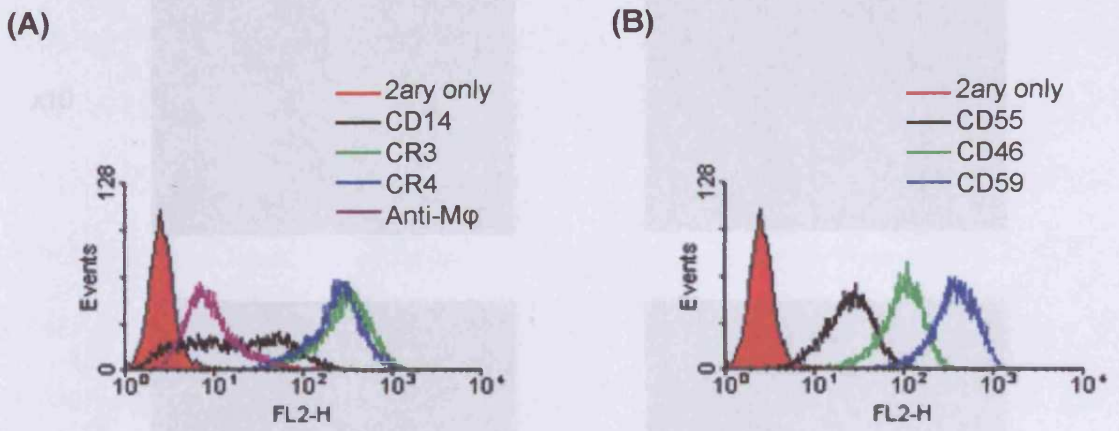


Figure 3.18: Expression of macrophage markers and CRegs on primary human macrophages after 7d differentiation from monocytes. **(A)** Macrophage markers. Primary macrophages express CD14 and bind anti-macrophage mAb (anti-Mφ), confirming that they are mature. The cells also express CR3 and CR4, indicating that they have the receptors required for C-mediated phagocytosis. **(B)** Primary macrophages express the CRegs CD55, CD46 and CD59.

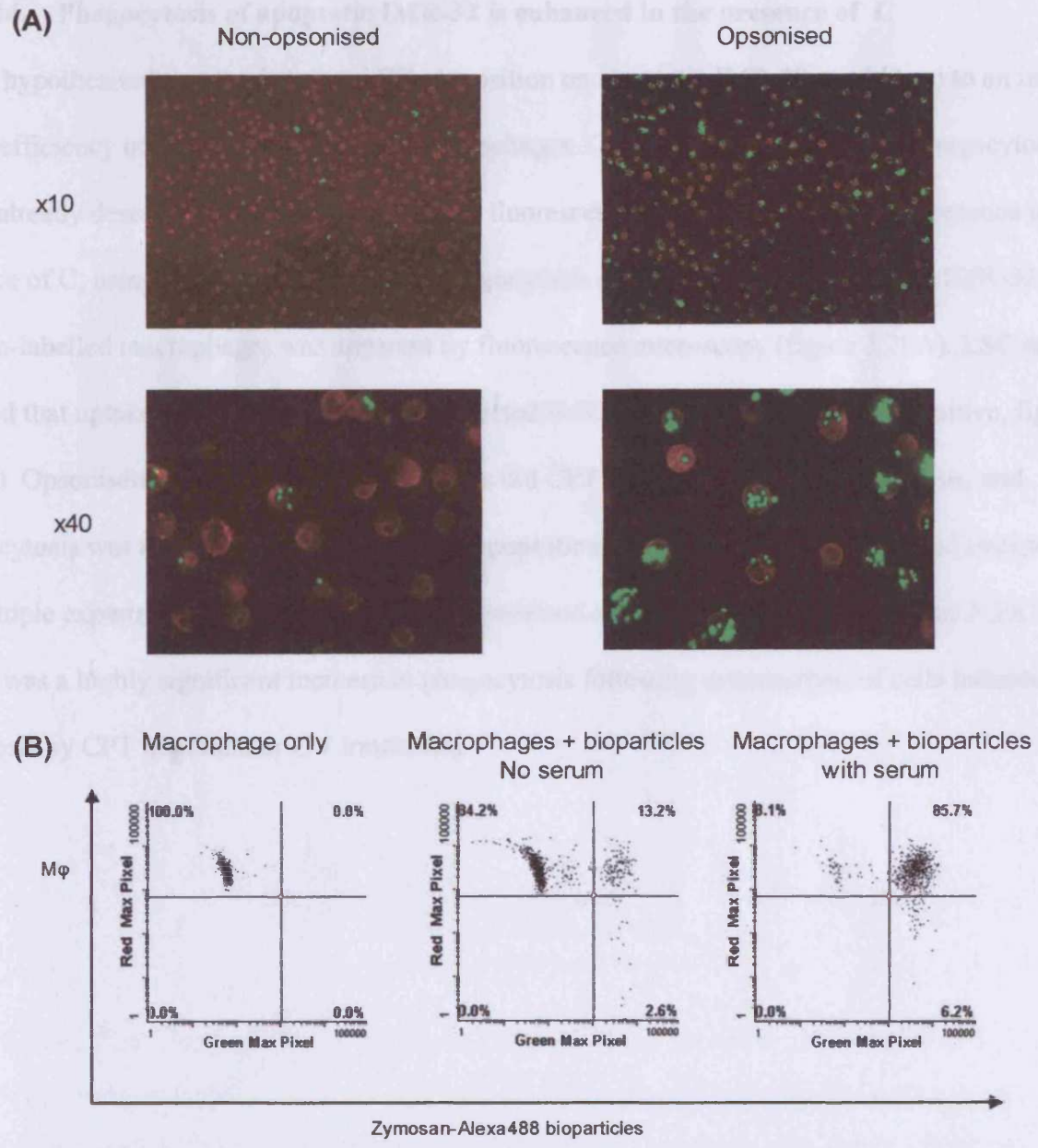


Figure 3.19: Primary human macrophages are capable of phagocytosis and this is enhanced in the presence of C. (A) Fluorescence microscopy of macrophages (red; PKH26 labelled) phagocytosing zymosan-Alexa488 bioparticles (green) with and without NHS as a source of C. There is a clear increase in phagocytosis in the presence of C, with more macrophages phagocytosing, and more particles phagocytosed per macrophage. **(B)** LSC analysis of phagocytosis. The number of macrophages phagocytosing bioparticles is shown. A clear increase is seen in the presence of C.

3.14. Phagocytosis of apoptotic IMR-32 is enhanced in the presence of C

It was hypothesised that the increased C3b deposition on apoptotic IMR-32 would lead to an increase in the efficiency of cells being cleared by macrophages. Using a modification of the phagocytosis assay already described, macrophages were fed fluorescence labelled IMR-32 in the presence or absence of C, using C8d as the source. The phagocytosis of PKH26-labelled apoptotic IMR-32 by calcein-labelled macrophages was apparent by fluorescence microscopy (figure 3.20A). LSC analysis showed that uptake of non-apoptotic, non-opsonised IMR-32 was inefficient (3.9% positive, figure 3.20B). Opsonisation increased phagocytosis, as did CPT and UV induction of apoptosis, and phagocytosis was most efficient for opsonised apoptotic cells. These data were analysed statistically in multiple experiments to compare uptake of opsonised and non-opsonised cells (figure 3.20C). There was a highly significant increase in phagocytosis following opsonisation of cells induced to apoptosis by CPT treatment or UV irradiation.

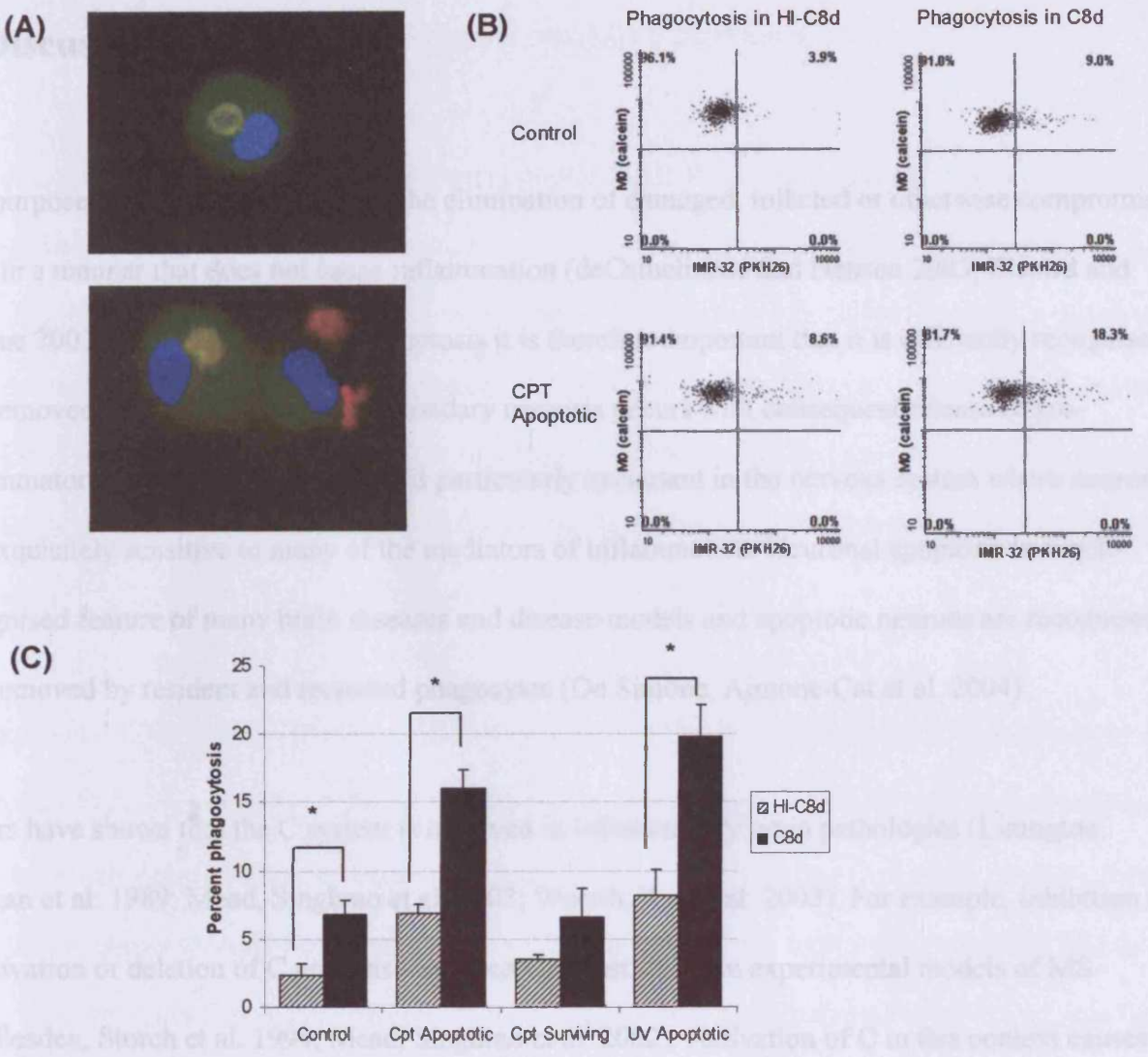


Figure 3.20: Phagocytosis of apoptotic IMR-32 in the presence and absence of C. (A) Macrophages phagocytose apoptotic IMR-32. Macrophages were labelled with calcein (green), and IMR-32 were labelled with PKH26 (red). Apoptotic cells can be observed within the macrophages (yellow). Magnification x40. **(B)** Laser scanning cytometry (LSC) of phagocytosis. For phagocytosis, only green fluorescence events were recorded and the maximum red fluorescence was recorded within these events to measure phagocytosis as percent of total macrophages. Cells in the upper right quadrant have phagocytosed one or more IMR-32. **(C)** Phagocytosis of control and apoptotic IMR-32. Apoptotic IMR-32 were more readily phagocytosed than controls, and this was further enhanced by the presence of C. Cells surviving CPT treatment were not readily phagocytosed. * $p < 0.01$. Results are representative of three experiments.

4. Discussion

The purpose of apoptosis is to enable the elimination of damaged, infected or otherwise compromised cells in a manner that does not cause inflammation (deCathelineau and Henson 2003; Elward and Gasque 2003). When a cell dies by apoptosis it is therefore important that it is efficiently recognised and removed by phagocytes before secondary necrosis occurs with consequent release of pro-inflammatory mediators. This process is particularly important in the nervous system where neurons are exquisitely sensitive to many of the mediators of inflammation. Neuronal apoptosis is a well-recognised feature of many brain diseases and disease models and apoptotic neurons are recognised and removed by resident and recruited phagocytes (De Simone, Ajmone-Cat et al. 2004).

Others have shown that the C system is involved in inflammatory brain pathologies (Linington, Morgan et al. 1989; Mead, Singhrao et al. 2002; Weerth, Rus et al. 2003). For example, inhibition of C activation or deletion of C proteins can protect against injury in experimental models of MS (Piddlesden, Storch et al. 1994; Mead, Singhrao et al. 2002). Activation of C in this context causes myelin damage and axonal injury and inhibition of C prevents these pathologies. However, C has protective roles both in defence against pathogens and in the clearance of toxic debris, including apoptotic cells (Takizawa, Tsuji et al. 1996; Mevorach, Mascarenhas et al. 1998; Taylor, Carugati et al. 2000; Ogden, deCathelineau et al. 2001). In this chapter I wished to address whether C activation in the brain might contribute to the efficient clearance of apoptotic neurons and the resolution of injury, or promote a pro-inflammatory phenotype by sensitisation of apoptotic neuronal cells to C-mediated lysis. To test this the well-characterised human neuroblastoma line IMR-32 was chosen, since this cell line has retained the C activating features of primary neurons, and expresses the same membrane CReg, CD46 and CD59, but not CD55 (Gasque, Thomas et al. 1996; Singhrao, Neal et al. 2000; van Beek, van Meurs et al. 2005). Apoptosis was induced using both chemical and physical

agents and confirmed using biochemical and morphological techniques.

Loss of CReg from neuronal cells undergoing apoptosis was found with a variety of induction agents, and confirmed using time course and double staining studies. Both CD46 and CD59 were lost from apoptotic IMR-32 cells, although the kinetics of loss was different, with CD46 cell surface expression reduced earlier than CD59. Loss of CReg also occurs during neutrophil apoptosis (Jones and Morgan 1995), and decreased expression of CD59 on apoptotic CD8+ T cells has been shown in autoimmune disease (Tsunoda, Kawano et al. 2000). However, neither of these studies addressed the mechanism of loss.

The temporal difference in CD46 and CD59 loss indicated that their mechanism of loss may be different. CD59 was present in both the soluble fraction of the supernatant and also in the particulate fraction, which suggested that it was cleaved into a soluble form, and also present on apoptotic blebs. Indeed, the soluble form had an apparent Mr 2-3kDa lighter than the form in the cell lysate and on blebs, supporting the hypothesis that it was cleaved, but MMPs did not appear to be involved in this process. Others have shown that CD59 is shed on vesicles following CPT treatment of endothelial cells, even at doses insufficient to induce apoptosis (Simak, Holada et al. 2002), and it has previously been shown that CD59 and CD55 are present on ectosomes, microparticles shed by T cells and involved in antigen presentation, where they are important in protection against C-mediated lysis (Clayton, Harris et al. 2003). It is possible that events placing GPI anchored molecules on vesicles all occur via a common mechanism, and may have an important and common function in protecting blebs and other shed particles from C attack.

CD59 expression was also noted to be higher on cells surviving CPT treatment than on control cells. This was not seen with other the other induction agents investigated, most likely as cells entered

apoptosis at roughly the same time with these agents, unlike with CPT treatment which induces apoptosis mainly in cells in S-phase (Johnson, Ng et al. 1997), but also damages cells in other phases of the cell cycle (Morris and Geller 1996). A similar pattern of increased expression on surviving cells and reduced expression on apoptotic cells was seen with the immune modulator CD200, suggesting that CD59 and CD200 may have a similar mechanism of regulation. CD200 has been shown to be regulated by p53 in apoptosis-associated immune tolerance (Rosenblum, Olasz et al. 2004), and recently a study in our group has identified p53 as a regulator of CD59 expression using the IMR-32 model described here (Donev, Cole et al. in press).Thus IMR-32 cells exposed to agents that induce p53 such as CPT may undergo a "threshold" test, where cells with irreparable damage undergo apoptosis and lose CD200 and CD59, resulting in a pro-clearance phenotype where cells activate phagocytes via loss of CD200, and shed apoptotic blebs protected by CD59. Cells with more minor damage also activate p53, but this results in increased p53 methylation and subsequent activation of repair and survival mechanisms, with the cell inducing more CD200 to prevent phagocytosis and dampen the immune response in the immediate environment, and more CD59 to promote resistance to C-mediated killing.

In contrast to CD59, CD46 is shed from apoptotic cells as a soluble form only. This was due in large part to the activity of MMPs, particularly MMP-3, MMP-8 and MMP-9, and resulted in truncated soluble forms. The involvement of MMP-8 was surprising, since this MMP is produced mainly by neutrophils and chondrocytes. However, it has recently been shown that cells of neural crest origin also express MMP-8 during development (Giambernardi, Sakaguchi et al. 2001), a finding that is in keeping with the use of a neuroblastoma cell line. It remains to be established whether mature neurons also express this MMP.

The MMP-mediated mechanism of CD46 shedding is also exploited in other contexts. For example,

Loxoceles spider venom activates MMPs causing loss of CD46 (Van Den Berg, De Andrade et al. 2002). Importantly MMPs have been implicated both in neurodegenerative processes and in apoptosis (Rosenberg 2002), and of particular relevance here, MMPs/ADAMs have been shown to have a role as "sheddases", cleaving death receptors, death-inducing ligands, and adhesion molecules from the surface of neurons (Hubschmann, Skladchikova et al. 2005). MMPs, such as MMP-3, can also act as stimulatory signals for microglia when released by apoptotic neurons (Kim, Kim et al. 2005), and MMP inhibitors have protective effects in neuroinflammatory diseases such as EAE (Hewson, Smith et al. 1995; Clements, Cossins et al. 1997). The shed soluble CD46 may also have a protective, anti-inflammatory role in the environment surrounding the apoptotic cells by controlling local C activation, perhaps even after the dying cell has been cleared. Indeed, in studies of CD46 cleaved by MMPs from tumour cells, CD46 was shown to retain its cofactor activity (Hakulinen, Junnikkala et al. 2004).

The loss of CD46 and CD59 from apoptotic neuronal cells, coupled with their capacity to spontaneously activate C, could result in a cell surface where C activation accelerates in an uncontrolled manner. Deposition of C activation products, such as C3b, was increased on apoptotic cells, but was not due to increased C1q binding, which only occurred on cells that had progressed to the late phase of apoptosis or necrosis, a finding in keeping with other studies (Gaipl, Kuenkele et al. 2001; Zwart, Ciurana et al. 2004). MBL has also been shown to bind to apoptotic cells and activate C, and like C1q this also occurs on late apoptotic cells (Nauta, Raaschou-Jensen et al. 2003). Although the data do not rule out a role for the lectin pathway, this route of activation has been shown to have a negligible role in opsonisation of apoptotic cells with C fragments in serum (Nauta, Raaschou-Jensen et al. 2003). This suggested that the loss of CD46 was the major contributor to the observed increase in C3b deposition, and this contention was supported by double staining experiments for C3b and CD46. The consequence of increased C activation and deposition for the

cell was then investigated. Either the cell would be more susceptible to MAC-mediated lysis and worsen inflammation, or be opsonised and efficiently cleared by phagocytosis and thus limit inflammation.

The next set of studies showed that CPT treated cells bound more MAC after 24h of treatment, but surprisingly were more resistant to C-mediated lysis at this time point. Changes in CD59 expression were apparent by 24 hours and it is possible that increased MAC binding reflected the decreased CD59 expression on the apoptotic population. In these initial experiments, mixed populations of apoptotic cells and surviving cells were used, so the observed resistance to lysis was further investigated using separate populations of apoptotic versus surviving cells, using lysis conditions equivalent to those used for the C deposition assays. Under these conditions, apoptotic cells after 12h CPT were more susceptible to lysis, but after 24h were more resistant to killing compared with controls. UV treated cells were also more susceptible to killing, implying that an acute pro-apoptotic insult results in a more pro-inflammatory phenotype, a response that could lead to a worsening of an inflammation and neuronal damage in the brain. Several reports have also described an increase in susceptibility to C lysis in apoptotic cells, such as neutrophils and Jurkat (Jones and Morgan 1995; Attali, Gancz et al. 2004). These results support the contention that increased susceptibility to lysis is a feature common to many cell types undergoing apoptosis.

In contrast to apoptotic cells after 12h CPT or 4h UV, cells surviving in the face of CPT treatment were found to be more resistant to C-mediated lysis. Of note, surviving cells at 24 hours had higher levels of CD59 than controls, which may explain their increased resistance to lysis. However, surviving cells at 12 hours, which had normal CD59 levels, and apoptotic cells at 24 hours, which had low CD59 levels, were also more resistant to C-mediated killing, suggesting that these cells express intrinsic lytic resistance mechanisms, independent of CD59. The phenomenon of CReg-independent

resistance to C lysis has previously been reported, particularly in the context of accommodation in transplantation (Koch, Kanazawa et al. 2005), and in the phenomenon of sublytic C attack priming cells to resist lytic C attack (Reiter, Ciobotariu et al. 1992). Thus CPT treatment induces a variety of changes in lytic susceptibility, including CReg dependent and independent lysis resistance mechanisms.

CD46 loss leading to increased C activation was the most likely mechanism explaining the increased susceptibility to lysis observed in apoptotic cells. The potentially damaging consequence of increased lysis was reduced by the use of MMP inhibitors, clearly demonstrating that these compounds are neuroprotective under these circumstances in vitro. This suggests that one of the reasons that MMP inhibitors are effective in animal models of neuroinflammatory disease such as EAE (Hewson, Smith et al. 1995), or excitoxicity induced using kainic acid (Campbell, Finlay et al. 2004) is that they block CReg loss from cells undergoing apoptosis and thus limit C mediated damage.

Phagocytosis and clearance of apoptotic debris is the other major outcome of C activation on apoptotic cells. A phagocytosis assay using human macrophages was set up and validated for this purpose, and confirmed that opsonisation of zymosan particles with C activation products enhanced phagocytosis. This system was used to examine phagocytosis of apoptotic IMR-32, and the data suggested that C activation on these cells was acting to reduce the load of apoptotic cells by promoting their clearance, and thus limit inflammation. This is in keeping with other published data describing enhanced phagocyte removal of C opsonised apoptotic targets (Takizawa, Tsuji et al. 1996; Mevorach, Mascarenhas et al. 1998). C1q has been reported to be the most physiologically relevant C opsonin for removal of apoptotic cells in most tissues in vivo (Taylor, Carugati et al. 2000). However, in the context of inflammation, clearance of apoptotic cells may become increasingly dependent on other arms of the C system to promote more efficient disposal.

The data presented in this chapter here suggest that apoptotic neuronal cells, stripped of the capacity to regulate C, activate C more readily, which results in a situation where the inflammatory response is on a knife-edge, balanced between efficient clearance of apoptotic debris and increased susceptibility to lytic cell death. Thus neuronal apoptosis may be an important determinant of inflammation in the brain: brisk clearance dampens inflammation, but excess apoptosis likely worsens damage as cells are lysed by C. It is possible that neuronal apoptotic death is more pro-inflammatory than apoptotic death in other cell types: CD200 is down-regulated on neuronal cells during apoptosis, but in dendritic cells is up-regulated, which in the latter case dampens inflammation and promotes immune tolerance (Rosenblum, Olasz et al. 2004). In contrast, neuronal cells that survive in the face of toxic stimuli induce mechanisms that may dampen microglial activation by upregulation of CD200, and protect the cell against C by increasing CD59 expression. These responses may help limit cell loss, and promote resolution and repair. In terms of the hypothesis framed in the introduction, neuronal apoptosis is not predominantly anti-inflammatory, but the response induced in surviving cells does appear to be. These findings have implications for the use of C therapeutics in neurological disease, since both MMP-inhibitors and therapeutic CReg that target C activation could help attenuate inflammation and limit damage, but may also impair the clearance of dying cells and exacerbate disease.

Chapter 4: Consequences of lytic and sublytic MAC attack on neuronal cells

1. Introduction

The terminal pathway of the C system results in the formation of the MAC, a structure that forms a pore in the membrane of the cell. The MAC has been implicated as the cause of damage in a variety of inflammatory diseases, including those affecting the CNS such MS (Mead, Singhrao et al. 2002) and stroke (Huang, Kim et al. 1999). As a result a number of therapeutics are being designed with control of MAC formation in mind, since this leaves beneficial effects of C activation intact, such as opsonisation, while tackling its most damaging product.

However, the role of the MAC may not be so straightforward. Nucleated cells are relatively resistant to lysis by the MAC compared to erythrocytes, and cells may therefore survive a certain level of MAC in their plasma membrane, so-called sublytic attack (Morgan 1989). In many cell types the phenomenon of "induced protection" is seen, where cells exposed to a sublytic dose of MAC are more resistant to subsequent lytic C attack (Reiter, Ciobotariu et al. 1992; Reiter, Ciobotariu et al. 1995). Most studies to date on this phenomenon have been done on leukaemia cell lines, although other cells also display these effects. Induced protection appears to be an active process, dependent on RNA and protein formation (Jurianz, Ziegler et al. 1999), but little is known about mechanisms and no studies have addressed its role in neuronal cells, where it may help resist the damaging effects of C.

Studies in oligodendrocytes and Schwann cells have demonstrated that sublytic MAC can protect against apoptosis and promote entry into the cell cycle (Rus, Niculescu et al. 1997; Soane, Rus et al. 1999; Dashiell, Rus et al. 2000). MAC has been shown to activate survival pathways in these cells involving modulation of Bcl-2 and caspase-3, and also activation of PI-3-kinase pathways (Soane, Rus et al. 1999; Soane, Cho et al. 2001). These findings have been extended in vivo, and studies using C5 deficient mice with EAE have shown that in the absence of C5 pro-apoptotic genes are up-regulated, and repair processes, such as remyelination, in the chronic phase of disease are impaired (Niculescu, Weerth et al. 2004). In kainic acid treated C5 deficient mice, more neuronal damage was seen, and altered electrophysiology was noted (Pasinetti, Tocco et al. 1996). This suggests a role for C5 in neuroprotection and neuroregeneration, but in vivo studies in C5 deficient mice do not distinguish between the effects of the MAC and C5a.

Neuronal cells spontaneously activate C via the classical pathway, and are sensitive to C-mediated lysis due to relatively low levels of CRegs compared to glial cells (Gasque, Thomas et al. 1996; Singhrao, Neal et al. 2000). Little is currently known about the effects of sublytic levels of the MAC on these cells, a phenomenon that may be of importance in neuroprotection by resisting further C attack, and in preventing neuronal loss by apoptosis. This part of the thesis aimed to address these issues in vitro.

The hypotheses are:

1. **Neuronal cells have intrinsic mechanisms to protect against MAC attack other than classical CReg**

2. Sublytic MAC attack protects neuronal cells from cell death by lytic C attack and apoptosis, and modulates the immune response accordingly

The study was carried out in vitro using the IMR-32 neuroblastoma cell line, which is similar to primary human neurons in its CReg profile and in its capacity to spontaneously activate C via the classical pathway.

2. Specific methods

2.1. Reactive lysis

C56 was generated using a freeze-thaw method. Equal quantities (200μg) of C5 and C6 were mixed together on ice in 1ml CFD. The mix was kept at 4°C for 24 hours and then frozen at or below -20°C for at least 24 hours before use. Just before use, C56 was thawed at 37°C for 30 minutes and used within 3 hours.

For reactive lysis, C56 was added to cells in the appropriate quantity (1-20μg) in 100μl CFD and incubated for 5 minutes at 22°C. C7 (300ng) was then added to the cells and incubated at 37°C for 15 minutes. Cells were then washed once in 200μl CFD and incubated in 100μl CFD containing 200ng each of C8 and C9 for 60 minutes at 37°C.

Alternatively, cells were incubated with C8d serum (1-20%) diluted in CFD for 15 minutes at 37°C to generate C5b-7 sites. Cells were then washed twice in CFD, and incubated in 100μl CFD containing 200ng each of C8 and C9 for 60 minutes at 37°C.

2.2. Fluorescence plate reader calcium flux assay

Cells were harvested and resuspended to $1x10^6$/ml in serum free medium. Cells were loaded with Fura-2.AM (2μM of 1mM DMSO stock) diluted in serum-free medium for 45 minutes. Cells were then washed twice in CFD with 1.3mM $CaCl_2$ and stored on ice until analysis.

The FluoStar Optima fluorescence plate reader was used with the plate temperature set at 37°C, and a

protocol used with dual filters set at 340nm excitation/ 510nm emission and 380nm excitation/ 510nm emission.

To check fura-2 loading and to calculate Ca^{2+} concentrations Rmin and Rmax ratios (340nm/380nm) were obtained. CFD + 0.2% Triton X-100 + 5mM EGTA (100µl) was added to 1x 10^5 cells in 100µl (Rmin) and CFD + 0.2% Triton X-100 + 1.3mM $CaCl_2$ (100µl) was added to 1x 10^5 cells in 100µl (Rmax). Samples were agitated and incubated for 5 minutes at 37°C, and read on the plate reader at 37°C.

Samples were analysed using a time-resolved method, with measurements taken every 2 or 4 seconds for 10 to 15 minutes. Ca^{2+} concentrations were calculated using the following equation:

$$[Ca^{2+}] = Kd \times \frac{(test - Rmin)}{(Rmax - test)} \times \frac{Sf_2}{Sb_2}$$

Where Sf_2 = 380nm value from Rmin (f=free), and Sb_2 is the 340nm value from Rmin (b=bound). Kd = dissociation constant for fura-2 binding to Ca^{2+} = 224.

2.3. Single cell calcium imaging

Cells for analysis were plated out on poly-D-lysine coated coverslips in a six-well plate with 3 x 10^5 cells per well, and allowed to adhere overnight. Cells were loaded with Fura-2 by removing the medium and incubating with 200µl of 1µM Fura-2.AM in serum-free RPMI for 30 minutes at 37°C. Cells were gently washed in culture medium and coverslips were mounted on a heated microscope

stage (37°C). Single cell fluorescence was recorded using a CCD camera mounted on the microscope and acquired and analysed using Imagemaster software with the assistance of Dr MB Hallet (Dept Surgery, Cardiff University).

2.4. Electron microscopy of MAC-attacked liposomes

Liposomes were made by Dr S Tilley (Dept Structural Biology, Birkbeck College, London) by trituration of 10mM phosphotidylcholine in PBS as described (Tilley, Orlova et al. 2005). Liposomes (10µl) were then incubated with C56 (10µg) for 5 minutes at 22°C, and C7 (5µg) added for 10 minutes at 37°C. Finally, C8 and C9 (5µg each) were added to complete the reaction. Liposomes with MAC (3µl) were then negatively stained by applying the sample to a carbon-coated grid for 30 seconds, blotting the excess, and then applying 3µl uranyl acetate for 30 seconds, and blotting. Samples were then analysed on a transmission electron microscope with the assistance of Dr S Tilley (Birkbeck College, London).

3. Results

3.1. C activation and lysis of IMR-32 cells

IMR-32 neuroblastoma cells have been shown to spontaneously activate C via the classical pathway (Gasque, Thomas et al. 1996). The first experiments were therefore to characterise C activation on these cells in order to confirm and extend the findings of others. IMR-32 were exposed to various concentrations of C1q-FITC and binding was analysed by flow cytometry. Figure 4.1 shows that IMR-32 bound C1q in a dose dependent fashion, confirming that they may have the capacity to activate the classical pathway independent of antibody binding.

The pathway by which IMR-32 activate C was then interrogated by the use of 20% C8d serum diluted in various activation pathway buffers. C8d serum was used in order to minimise lysis at the high serum concentration used, which therefore allowed greater sensitivity and avoided artefacts from dead cell debris. CFD was used to allow optimal activation of all pathways, since it contains both Ca^{2+} and Mg^{2+}; veronal (barbitone) buffered saline (VBS) + 5mM EDTA was used to block both classical and alternative pathways by chelating calcium and magnesium; and VBS + 2mM $MgCl_2$ was used to allow activation of the alternative pathway only. Deposition of C3b was used as a marker of pathway activation. Figure 4.2 shows that only C8d serum diluted in CFD resulted in the deposition of C3b, with none seen in the presence of Mg^{2+} supplementation only, or when both pathways were blocked. HI-C8d also resulted in no C3b deposition. This confirmed that the alternative pathway is not involved in activation, and blockade of the both pathways together demonstrated that Ca^{2+} was required for activation of C on these cells, confirming that the classical pathway is the route of activation.

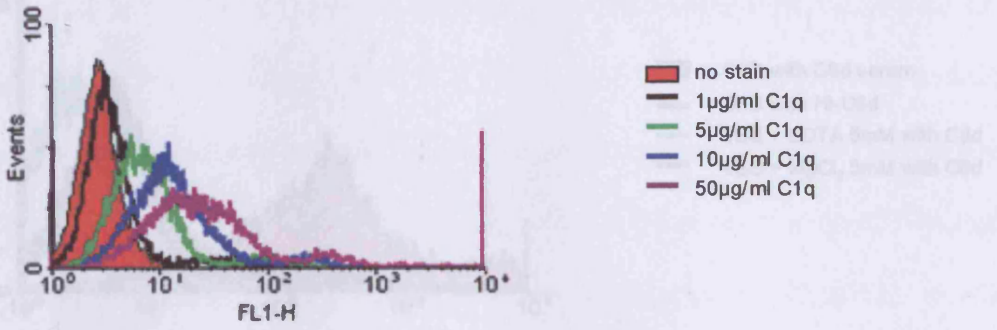


Figure 4.1: IMR-32 directly bind C1q. IMR-32 were harvested and incubated with varying concentrations of C1q-FITC for 30 minutes at 37°C, washed, and analysed on a flow cytometer. The results show dose-dependent binding of C1q directly to the surface of IMR-32 cells.

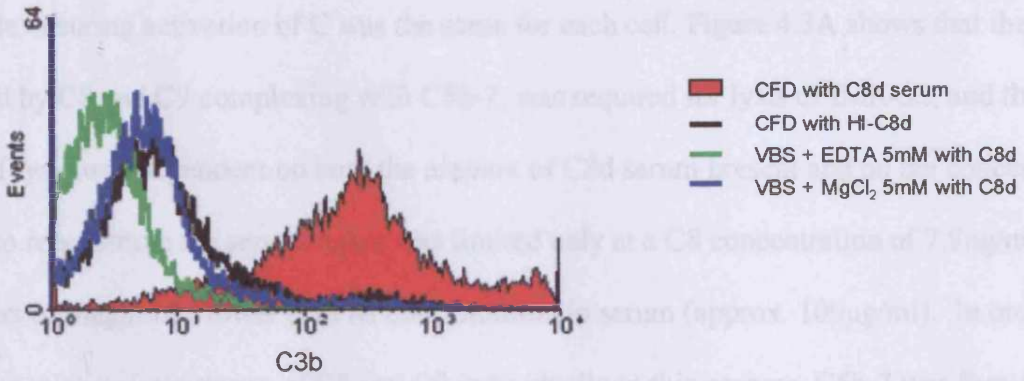


Figure 4.2: IMR-32 activate C via the classical pathway. IMR-32 were harvested and incubated with C8d serum in various buffers that either permit full activation of all C pathways (CFD), activation of the alternative pathway only (VBS+$MgCl_2$), or neither classical nor alternative pathways (VBS+EDTA). Cells were then stained with an anti-C3b antibody (C3/30) and analysed on a flow cytometer. Only cells incubated with C8d serum in CFD activated C to the C3b stage, indicating that the classical pathway was required for C activation on IMR-32.

A major consequence of C activation on cells is formation of the MAC and subsequent cell death by cytolysis. This was addressed in IMR-32 by analysing degree of cell death following incubation with C8d serum reconstituted with C8 at limiting concentrations, which allowed varying amounts of MAC to form while ensuring activation of C was the same for each cell. Figure 4.3A shows that the MAC pore, formed by C8 and C9 complexing with C5b-7, was required for lysis of IMR-32, and that efficiency of lysis was dependent on both the amount of C8d serum present and on the concentration of C8 used to reconstitute the serum. Lysis was limited only at a C8 concentration of 7.8ng/ml, several orders of magnitude lower than its concentration in serum (approx. 100μg/ml). In order to determine the relative importance of C8 and C9 individually in this process, C5b-7 was formed on the cells by incubating with C8d, and then washing the cells, and then adding either C8 alone or C8 and C9. Figure 4.3B shows that C5b-7 lysed <5% cells (background; 0ng C8 in figure), and the addition of 20ng C8 to form C5b-8 was capable of killing 6% cells ($p<0.05$). Formation of C5b-9 resulted in the most efficient lysis of IMR-32 (22% C-specific killing at 20ng C8; $p<0.01$).

3.2. Preparation of terminal pathway components and C56

The aim of this study was to examine the effects of sublytic MAC attack on IMR-32. In order to achieve this, it was decided that the use of reactive lysis using purified components of the terminal pathway of C would be the best system to use to confirm any effects, since it examines only the effects of MAC, avoiding interference from other C components and activation products, and any confounding variables arising from unknown serum factors. MAC components were obtained from laboratory stocks, or purified as described in Chapter 2. In order to initiate the formation of the MAC, C5b6 is required. A number of studies have found that it is possible to form this from purified C5 and C6 using a variety of physicochemical means, including freeze-thawing (Dessauer, Rother et al. 1984), acid activation (Hammer, Hansch et al. 1983), and oxidation using chloramine-T (Vogt, Zimmermann et al. 1992). These methods do not activate C5 in a physiological manner, since C5a is

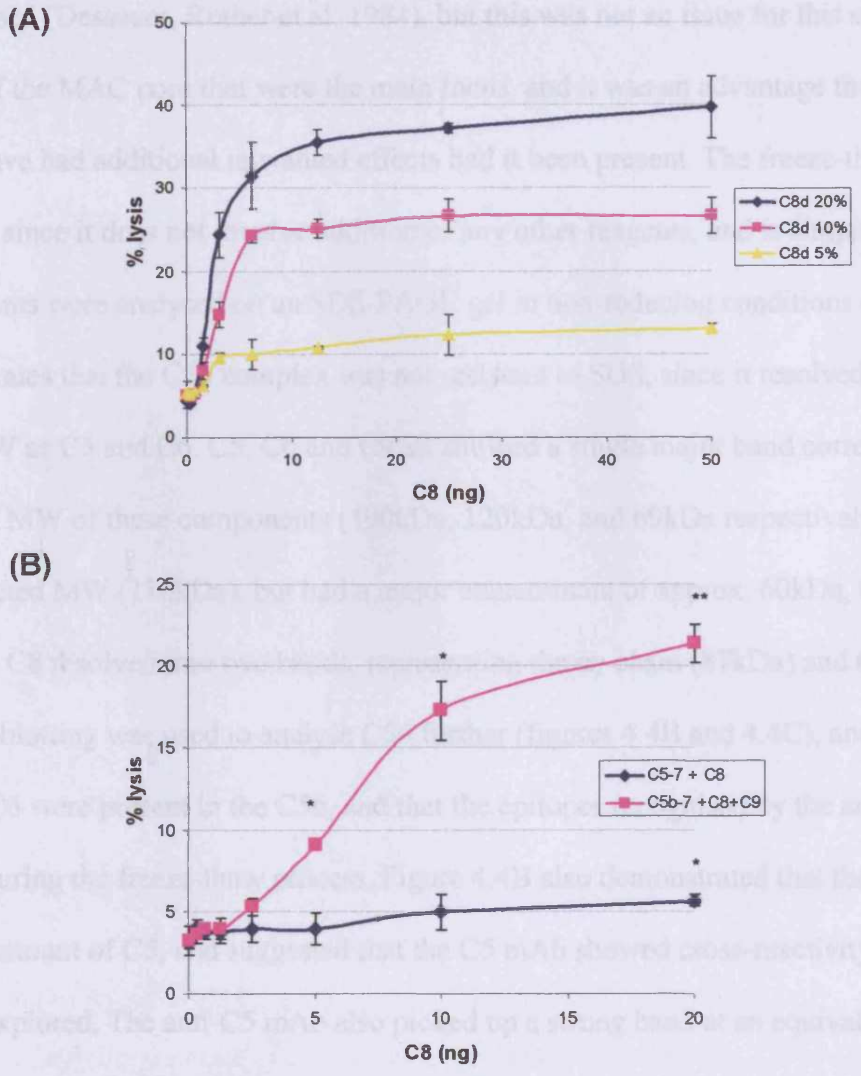


Figure 4.3: IMR-32 lysis is mediated via the MAC. (A) IMR-32 were incubated with C8d at various dilutions, reconstituted with amounts of C8, for 1 hour at 37°C. Cells were stained with PI and analysed by flow cytometry. Cytolysis showed dose dependence with both C8 and C8d serum dilution. Figure shows a representative experiment performed in triplicate +/- SD. **(B)** IMR-32 were harvested and incubated with C8d serum (10%) for 15 minutes to form C5b-7. Cells were then washed and incubated with various amounts of C8, either with or without C9 (200ng), for 1 hour at 37°C. Cells were stained with PI and analysed on a flow cytometer. The result showed that cells with C5b-7 (C8 0ng) had minimal lysis (3.5%), but C5b-9 treated cells showed clear lysis. C5b-8 formation only resulted in significant lysis with 20ng C8. Figure shows a representative experiment performed in duplicate +/- SEM. *$p<0.05$, **$p<0.01$, compared with 0ng C8 (C5b-7).

not released (Dessauer, Rother et al. 1984), but this was not an issue for this study since it was the effects of the MAC pore that were the main focus, and it was an advantage that C5a was absent, since it may have had additional unwanted effects had it been present. The freeze-thaw method was selected, since it does not involve addition of any other reagents, and is simple and reliable. The components were analysed on an SDS-PAGE gel in non-reducing conditions (Figure 4.4A), which demonstrates that the C56 complex was not resistant to SDS, since it resolved into two bands of the same MW as C5 and C6. C5, C6 and C9 all showed a single major band corresponding to the expected MW of these components (190kDa, 120kDa, and 69kDa respectively). C7 showed a band of the expected MW (110kDa), but had a major contaminant of approx. 60kDa, most likely to be albumin. C8 resolved into two bands, representing the αγ chain (87kDa) and the β chain (65kDa). Western blotting was used to analyse C56 further (figures 4.4B and 4.4C), and confirmed that both C5 and C6 were present in the C56, and that the epitopes recognised by the antibodies were not altered during the freeze-thaw process. Figure 4.4B also demonstrated that the purified C6 contained a small amount of C5, and suggested that the C5 mAb showed cross-reactivity with C6. This was not further explored. The anti-C5 mAb also picked up a strong band at an equivalent MW to C6, but in the C6 lane this was faint, suggesting that the C6 present in the C56 lane may have altered conformation allowing recognition by the anti-C5 mAb.

3.3. Reactive haemolysis and formation of MAC on liposomes

In order to assess whether the reactive lysis system was functional, haemolysis of unsensitised sheep E was used. The MAC was built sequentially as described in section 2.1. Figure 4.5 shows the haemolysis curve produced by reactive lysis. No lysis was seen with C5-7, but was clearly evident when the MAC was fully formed up to C5-9. Transmission electron microscopy was performed on liposomes attacked using reactive lysis. Figure 4.6 shows that MAC pores were formed on the surface of liposomes using this method, and were of the correct size (approx 10nm diameter). These

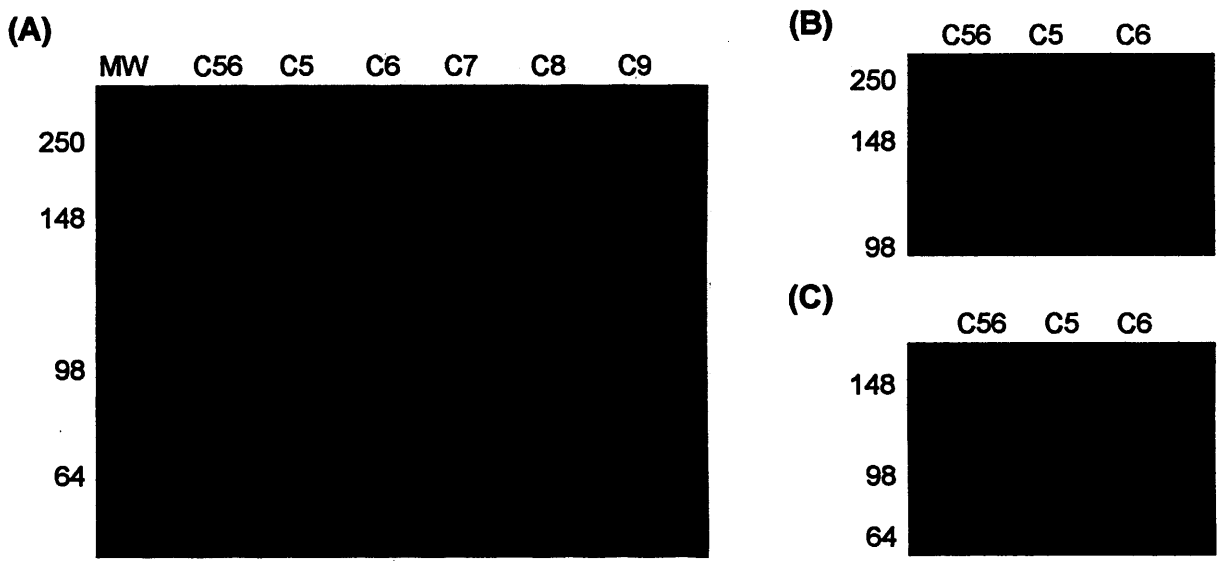


Figure 4.4: Characterisation of the terminal pathway components. Components and complexes were purified or obtained as described in Materials and Methods. **(A)** Coomassie stained 7.5% gel. All components showed a band of the correct MW. C7 was not as pure as the other components, possibly due to albumin contamination. C8 shows the two bands of the αγ chain complex and the β chain. The C56 complex formed using the freeze thaw method was not stable in the presence of SDS and dissociated into the two constituents C5 and C6. **(B)** Western blot using anti-C5 mAb. The results showed that C5 in the C56 complex is still recognised following the freeze-thaw procedure. A strong band was seen in the C56 lane at a similar MW to C6, but in the C6 lane this band was faint. **(C)** Western blot using anti-C6 mAb. Both C56 and C6 showed strong bands, indicating that the C6 epitope is still recognised following the C56 freeze-thaw procedure.

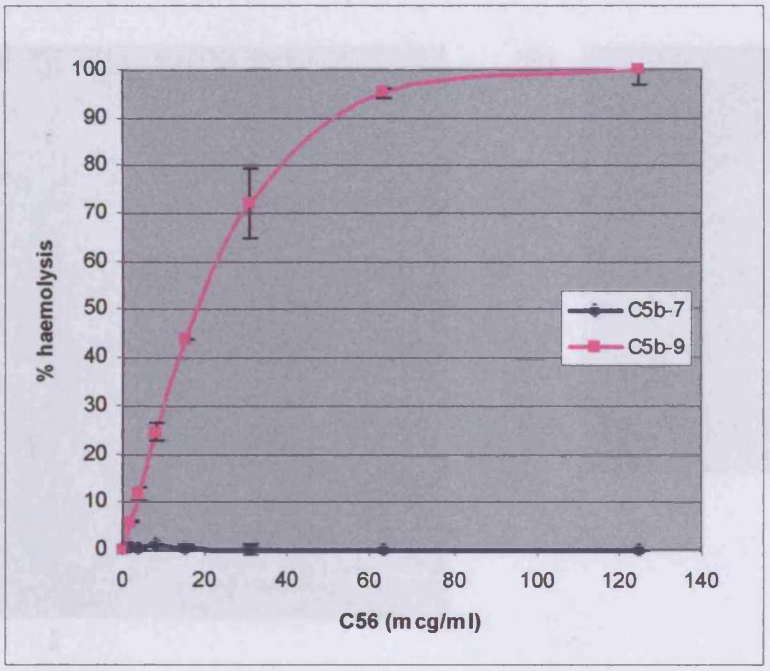


Figure 4.5: Reactive haemolysis using purified components. Unsensitised sheep E (2%) were incubated with varying concentrations of C56 in CFD for 5 minutes, followed by incubation with 200ng C7 for 15 minutes, and then incubation with 200ng each of C8 and C9 for 1 hour. 100% lysis was achieved with C5b-9, and no lysis was seen with C5b-7, indicating that the reactive lysis system was working.

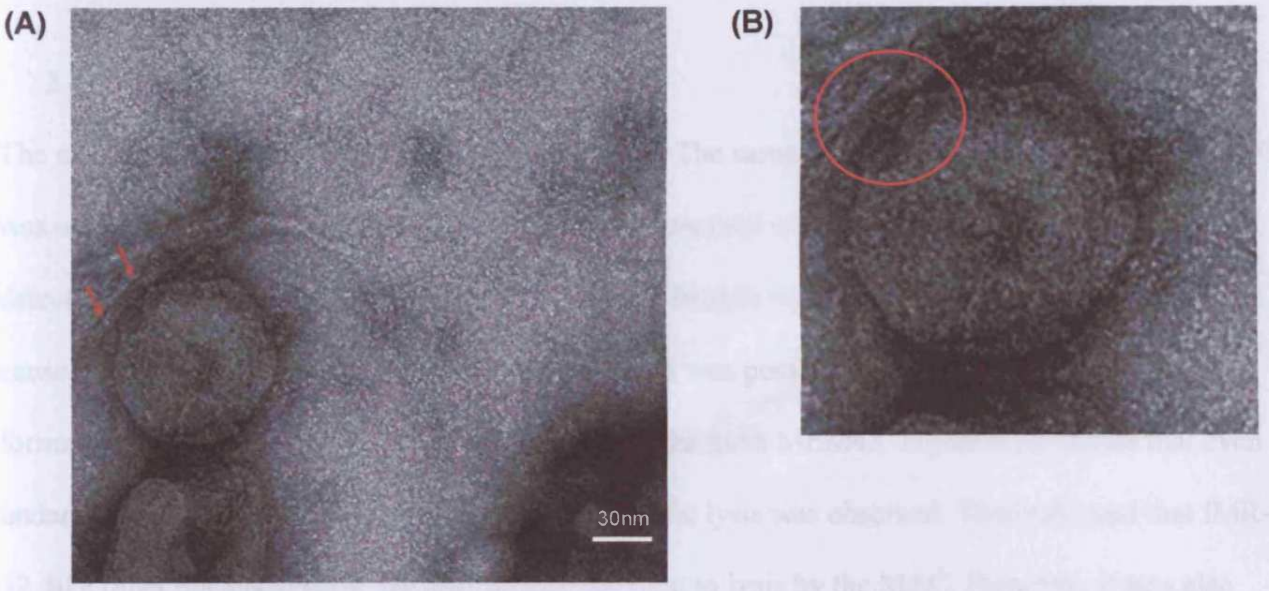


Figure 4.6: Electron microscopy of MAC formed by reactive lysis on liposomes.

MAC was formed on phosphotidylcholine liposomes using reactive lysis. Tubular side-on structures of approx 10-15nm across can be seen on a liposome (**A,** red arrows), and are magnified in **(B,** red circle**).**

experiments confirmed that the reactive lysis system was working, and that all components were active and produced a membrane pore.

3.4. IMR-32 are resistant to reactive lysis

The next step was to assess reactive lysis of IMR-32. The same protocol for construction of the MAC was used as for the haemolysis assay, and lysis was assessed using PI staining of dead cells and detection by flow cytometry. Figure 4.7A shows that despite using the same amount of C56 that caused 100% haemolysis (20μg), no lysis was seen. It was possible that CD59 was blocking the formation of the MAC, so CD59 was blocked using the mAb MEM43. Figure 4.7B shows that even under these conditions, a maximum of 5-6% C-specific lysis was observed. This indicated that IMR-32, like other nucleated cells, are intrinsically resistant to lysis by the MAC. However, it was also possible that the MAC was not correctly formed on these cells.

3.5. MAC is detectable on IMR-32 in the absence of lysis

In order to assess whether MAC was forming correctly on cells, mAb to C7 and C9 neoepitope (which detects only C9 present as part of the MAC) were used. IMR-32 were attacked using reactive lysis, and the presence of C7 and the MAC analysed by flow cytometry. Figure 4.8 shows that C5-7 attacked cells had C7 present on their cell surface, but no C9, but C5-9 attacked cells were positive for both C7 and C9, indicating that MAC was being formed sequentially and correctly, as expected.

3.6. Reactive lysis induces a reversible calcium flux in IMR-32

Although the MAC was present on the cell surface of IMR-32, it was not clear whether it was functional or not. To assess the cellular response to MAC formation, IMR-32 in suspension were loaded with the fluorescent ratiometric Ca^{2+} indicator dye fura-2.AM, and the C5-7 complex formed

(A) (B)

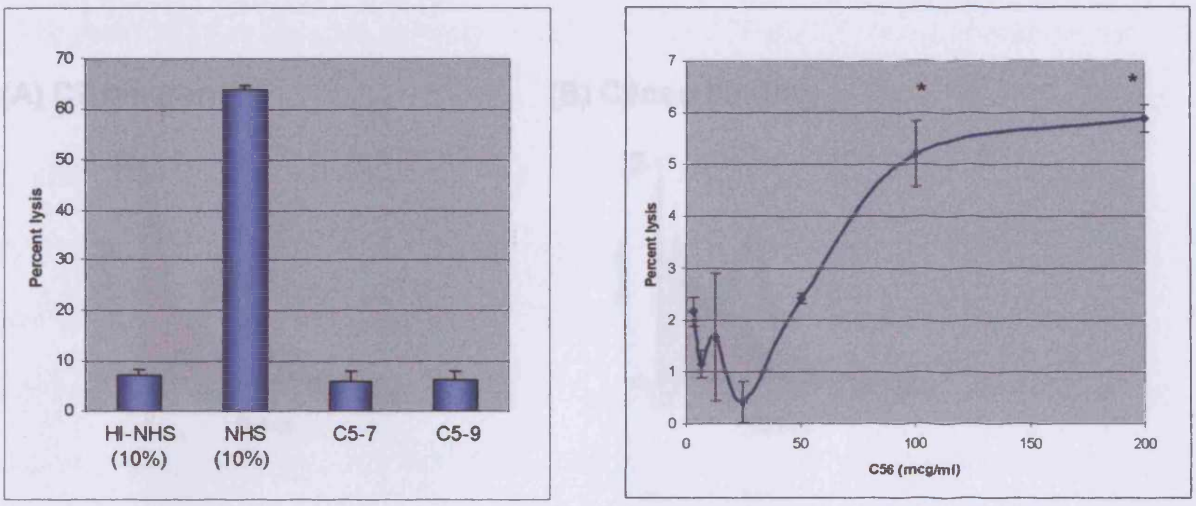


Figure 4.7: IMR-32 are resistant to reactive lysis with purified components. (A) A lysis assay using the reactive lysis system was performed on IMR-32 ($1x10^5$) , using PI to detect dead cells by flow cytometry. No IMR-32 lysis was detected following attack by maximal reactive lysis (C56 20µg). **(B)** Reactive lysis with blockade of CD59. After neutralisation of the main MAC inhibitor CD59 with the mAb MEM43, a small degree of lysis was noted, but reached only 6% maximum. Experiments were done in duplicate at least twice. Results expressed as mean +/- SD. * $p<0.05$ compared to C56 0mcg/ml.

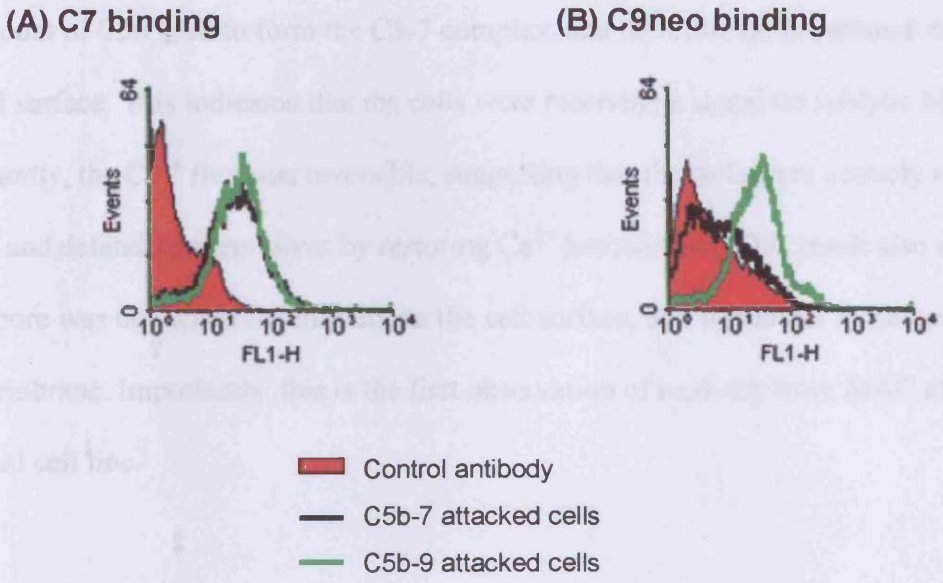


Figure 4.8: MAC is detectable on IMR-32 in the absence of lysis. Cells were exposed to MAC attack by reactive lysis up to the C5b-7 stage or the C5b-9 stage, and incubated with mAb to C7 **(A)**, or mAb to C9 neoepitope **(B)**. The results showed that both C5b-7 and C5b-9 attacked cells had C7 on the cell surface, but that only the C5b-9 attacked cells had formed the full MAC.

as described, before real-time analysis using a spectrophotometer. Figure 4.9A shows the Ca^{2+} flux induced following C8 and C9 addition at 60 seconds to IMR-32 that had C5-7 complexes on their surface. The peak Ca^{2+} flux occurred 2 minutes after addition of C8 and C9, and was dependent on the amount of C56 used to form the C5-7 complex, and therefore on the amount of MAC present on the cell surface. This indicated that the cells were receiving a signal on sublytic MAC attack. Importantly, the Ca^{2+} flux was reversible, suggesting that the cells were actively responding to MAC attack, and defending themselves by restoring Ca^{2+} homeostasis. This result also suggested that the MAC pore was not active indefinitely on the cell surface, and was either closed or removed from the cell membrane. Importantly, this is the first observation of recovery from MAC attack occurring in a neuronal cell line.

The increase in Ca^{2+} concentration in MAC attacked cells may have been due to either influx of extracellular Ca^{2+}, or release from intracellular stores. To address this, IMR-32 Ca^{2+} flux was studied in the presence of extracellular EGTA in a nominally Ca^{2+}-free buffer. Figure 4.9B shows that Ca^{2+} flux was abolished when EGTA was present, indicating that in this cell type, extracellular Ca^{2+} influx is required for generating the Ca^{2+} signal.

To examine the effects of MAC attack on Ca^{2+} flux on the single cell level, adherent IMR-32 were loaded with fura-2 and mounted on a heated microscope stage. Cells were then either attacked using reactive lysis, or with a lytic dose of C using NHS as a source. Figure 4.10A shows the Ca^{2+} flux profiles of four separate cells attacked with a lytic dose of C. A gradual increase in Ca^{2+} concentration was noted with all cells, but in three a threshold appeared to be reached, with a large increase in Ca^{2+} concentration which then persisted to the end of the experiment. Figure 4.10C (top row), shows the image of one of these cells, and in the final image at 15 minutes, lysis of the cell is evident as fura-2 escapes once membrane integrity is lost. Thus, in lytic C attack, Ca^{2+} influx precedes loss of

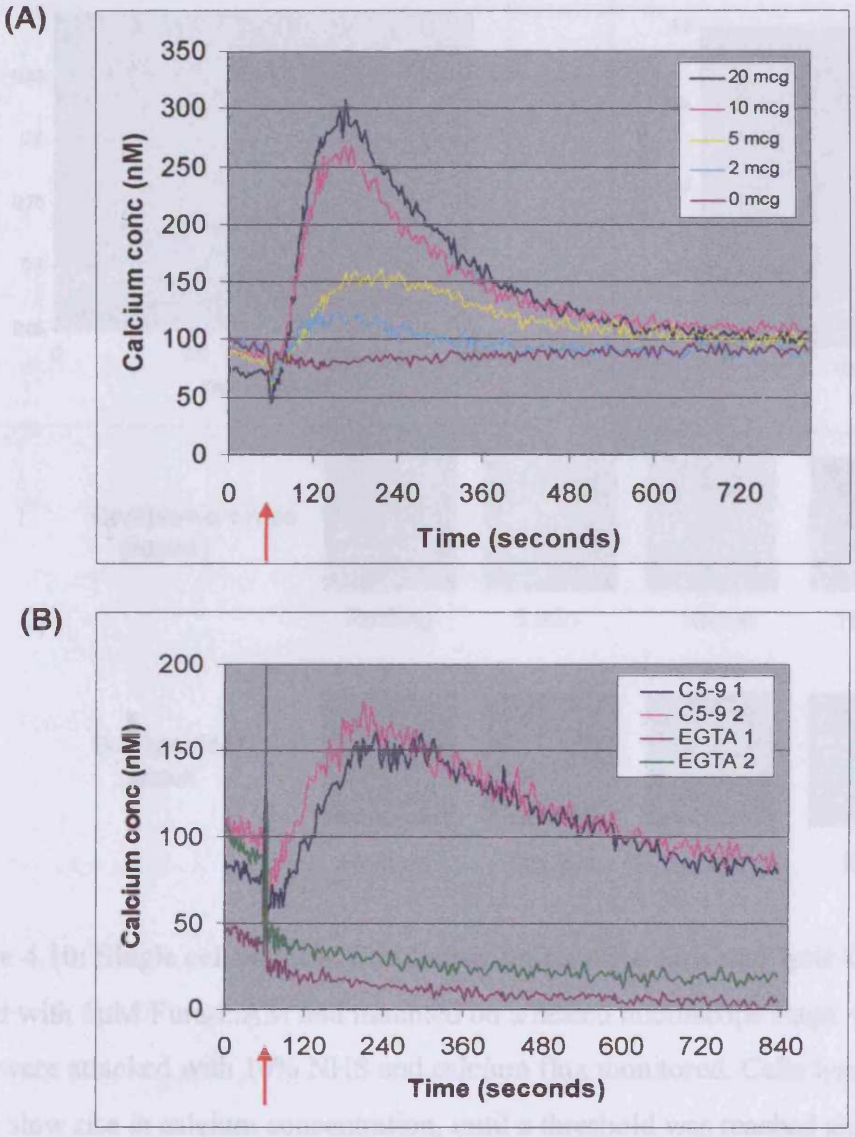


Figure 4.9: Sublytic MAC induces a reversible calcium flux in IMR-32. Cells were loaded with 1μM Fura-2.AM, and incubated with C56 and then C7 to form C5b-7. Cells were then washed and analysed on a spectrophotometer, and C8 and C9 injected onto the cells at 1 minute (red arrow). **(A)** Calcium flux in IMR-32 attacked using reactive lysis. IMR-32 ($1x10^5$) were incubated with C56 in the quantity indicated in the legend. Calcium flux was dependent on the dose of C56 used, and was reversible. **(B)** Calcium flux induced using reactive lysis is dependent on extracellular calcium. 5mM EGTA was used to chelate extracellular calcium. No calcium flux was observed under these conditions. Figure shows results from duplicate experiments for each condition.

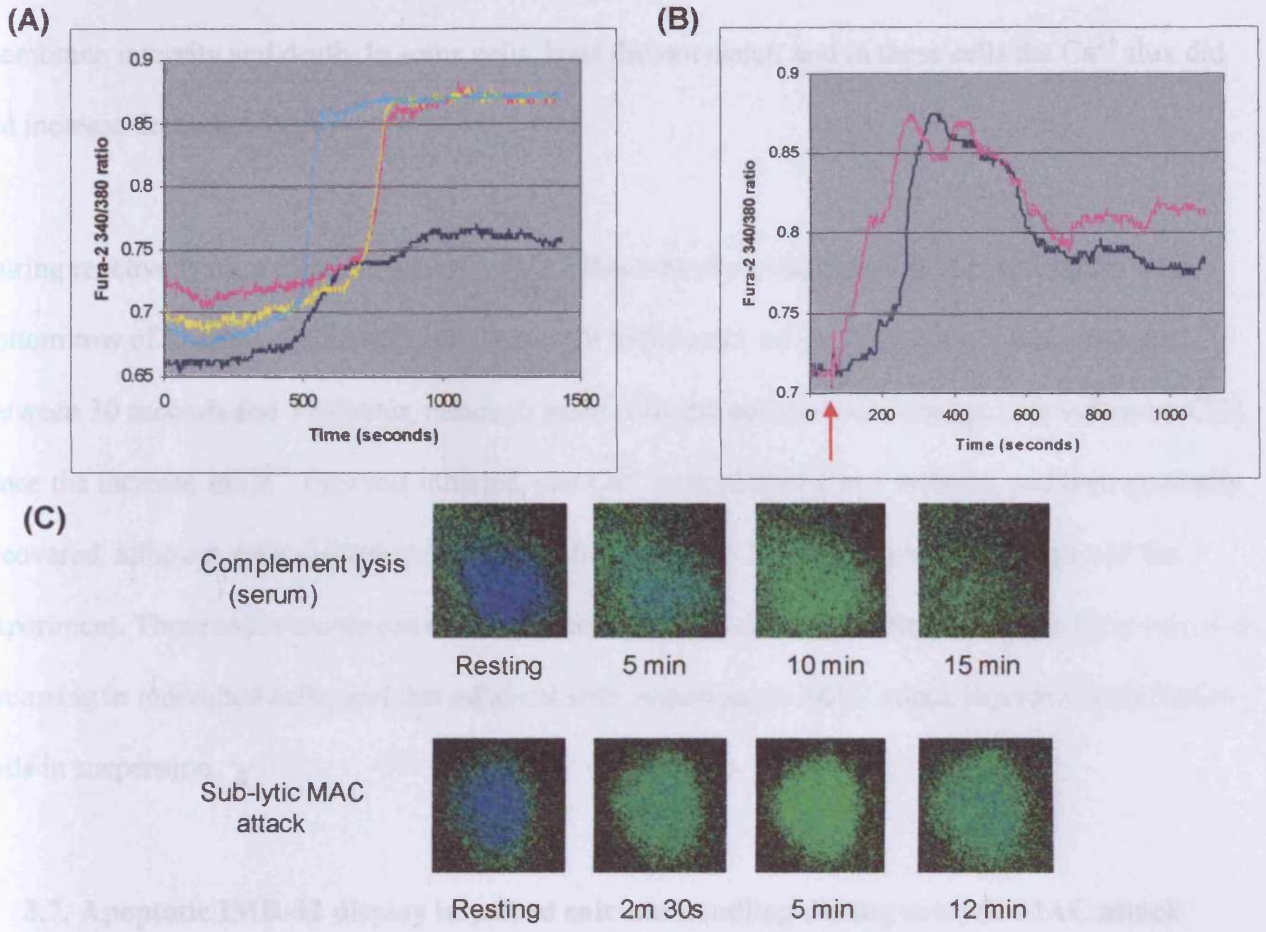


Figure 4.10: Single cell calcium flux following reactive lysis and lytic C attack. Cells were loaded with 1μM Fura-2.AM and mounted on a heated microscope stage. **(A)** Lytic C attack. Cells were attacked with 10% NHS and calcium flux monitored. Cells lysed by C showed an initial slow rise in calcium concentration, until a threshold was reached and a large increase in calcium occurred. These cells did not recover. Some cells (eg dark blue trace) remained viable, and showed smaller increases in intracellular calcium. **(B)** Reactive lysis. IMR-32 were incubated with C56 for 5 minutes, and then with C7 for 15 minutes. These components were removed and the cells incubated in CFD. C8 and C9 were added after 60 seconds (red arrow). Intracellular calcium increased shortly after addition of C8 and C9, reaching a peak after 2 minutes. Cells showed recovery, but not to the baseline level during the analysis period. **(C)** Single cell calcium images following lytic C attack and reactive lysis. Pseudocolour scale: dark blue indicates low calcium concentration, increasing through light blue and then green. The kinetics of attack were different, with reactive lysis calcium changes occurring quickly, and lytic C changes more gradual. Once cells were lysed, fura-2 became dispersed as the cell lost membrane integrity and the cell was no longer identifiable using this method (15 minutes lysis image).

membrane integrity and death. In some cells, lysis did not occur, and in these cells the Ca^{2+} flux did not increase dramatically.

During reactive lysis, a different pattern of Ca^{2+} flux was observed (figure 4.10B and figure 4.10C bottom row of images). On addition of C8 and C9 to the cells, an increase in Ca^{2+} flux was seen between 30 seconds and 3 minutes, although some cells did not show any change (see videos on CD). Once the increase in Ca^{2+} flux was initiated, cell Ca^{2+} peaked after 2 to 4 minutes, and then gradually recovered, although cells did not recover to the baseline Ca^{2+} level determined at the start of the experiment. These experiments confirmed that the Ca^{2+} flux observed at the population level was also occurring in individual cells, and that adherent cells responded to MAC attack in a similar fashion to cells in suspension.

3.7. Apoptotic IMR-32 display impaired calcium handling during sublytic MAC attack

Apoptotic IMR-32 are more susceptible to lytic C attack than normal controls (see Chapter 3). It was hypothesised that one reason for this may be an inability to efficiently handle calcium flux, since caspases active in apoptotic neurons have been found to cleave calcium channels (Schwab, Guerini et al. 2002). Apoptotic and control cells were loaded with fura-2.AM, incubated with C56 and then C7 to form C5-7 complexes, and analysed on a spectrophotometer, with the addition of C8 and C9 after 1 minute. Figure 4.11 shows that control cells recovered from the MAC-mediated calcium influx after 10 minutes. CPT treated cells, in contrast, had a higher peak calcium concentration following MAC attack, and did not recover calcium concentrations to the baseline during the experiment.

3.8. IMR-32 display "induced protection" in response to sublytic C attack

One well known consequence of sublytic C attack is "induced protection" to further challenge with a lytic dose of C or other pore former (Reiter, Ciobotariu et al. 1995). To assess whether this occurs in

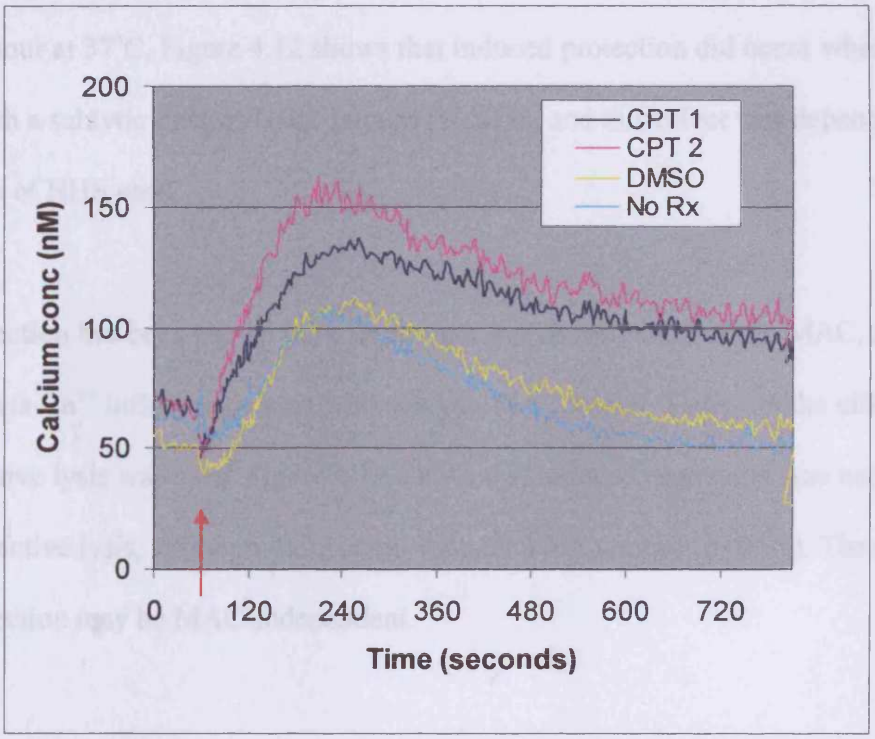


Figure 4.11: Calcium flux during sublytic MAC attack in apoptotic IMR-32. IMR-32 treated with CPT, DMSO (control) or no treatment for 12h, were were loaded with 1μM Fura-2.AM, and incubated with C56 and then C7 to form C5b-7. Cells were then washed and analysed on a spectrophotometer, and C8 and C9 injected onto the cells at 1 minute (red arrow). Control cells showed reversible calcium flux, with recovery after approx 10 minutes. CPT treated cells, in contrast, showed a higher peak calcium, and did not recover to baseline levels at 14 minutes. CPT 1 and 2 are duplicate experiments.

neuronal cells, IMR-32 were pre-incubated with a sublytic dose of NHS, titrated prior to the experiment, for 15 minutes at 37°C. Cells were then washed and exposed to a lytic dose of NHS (10%) for 1 hour at 37°C. Figure 4.12 shows that induced protection did occur when cells were pre-incubated with a sublytic dose of NHS, but not HI-NHS, and this effect was dependent on the concentration of NHS used.

Induced protection has been shown to be dependent on the formation of the MAC, and is thought to be mediated via Ca^{2+} influx associated with sublytic MAC attack. To assess the effect of MAC directly, reactive lysis was used. Figure 4.13 shows that induced protection was not seen with MAC formed by reactive lysis, although it did occur with the NHS control ($p<0.01$). These data suggest that induced protection may be MAC-independent.

To assess the MAC-independent effects in induced protection, IMR-32 were incubated with 10% C8d serum, with C8 reconstitution at concentrations around the sublytic point (see figure 4.3A). Induced protection was again observed (figure 4.14), but the addition of C8 had no further effect, confirming that MAC did not contribute to protection. Indeed, even when no additional C8 was present, a strong protective effect was seen with C8d serum compared with HI-C8d serum, with 50% less IMR-32 lysis ($p<0.001$). At the highest C8 dose used (30ng/ml), 5-10% C-specific lysis occurred during the pre-incubation step, which explains the increased lysis seen with C8d (figure 4.3A). These data suggest that induced protection is mediated by C, but is not dependent on the presence of the MAC pore.

3.9. Lytic C attack causes loss of CD200, CD46 and CD59

IMR-32 lose the immune regulator CD200, and the CRegs CD46 and CD59 during apoptosis (Chapter 3, figures 3.2-3.5). It was hypothesised that these cell surface molecules would also be

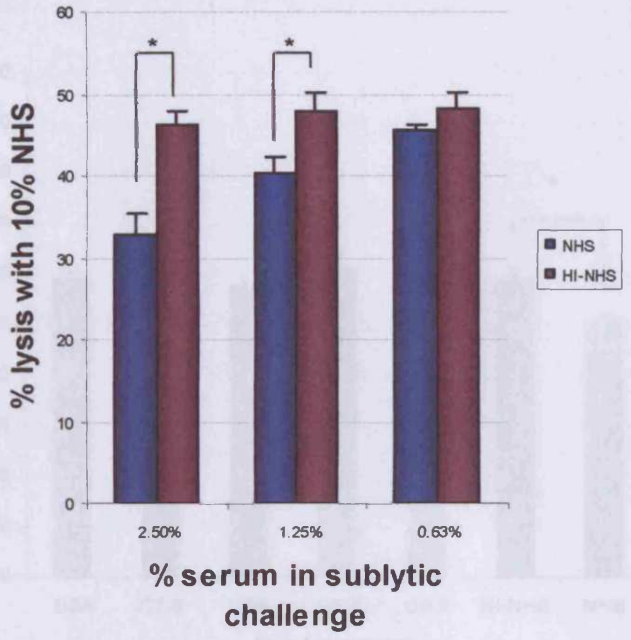


Figure 4.12: Sublytic C attack causes "induced protection" to lytic C attack. IMR-32 were incubated with sublytic dose NHS or HI-NHS in CFD for 15 minutes before exposure to 10% NHS (lytic dose) for 1 hour. Lysis was then assessed using PI staining and flow cytometry. Induced protection was evident following sublytic NHS pre-treatment, but did not occur with HI-NHS. The induced protection titrated out, and was minimal at a NHS concentration of 0.63%. * $p<0.01$.

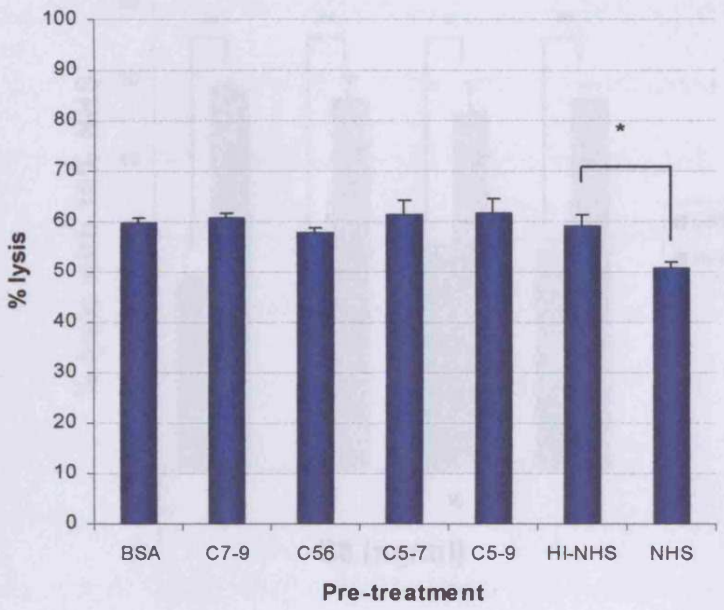


Figure 4.13: Reactive lysis does not cause induced protection to lytic C. IMR-32 were pre-treated with various combinations of reactive lysis MAC components as described in Materials and Methods, or with HI-NHS or NHS (2.5%) as controls, for 15 minutes. Cells were then washed once and incubated with 10% NHS for 1 hour, and lysis was analysed using PI and flow cytometry. Induced protection did not occur following reactive lysis, indicating that MAC was not involved in this process. Results are from an experiment performed in triplicate +/- SD. * $p<0.05$.

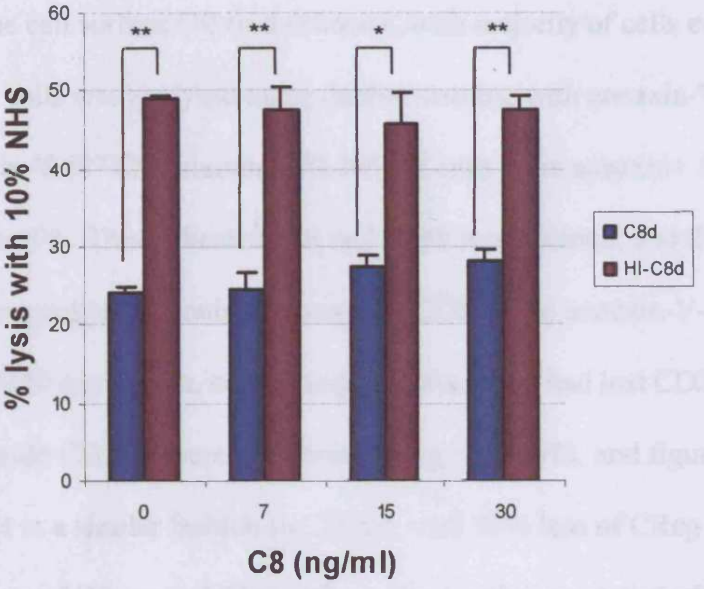


Figure 4.14: Induced protection is not MAC dependent. IMR-32 were incubated with C8d or HI-C8d (10%) in CFD reconstituted with varying amounts of C8 for 15 minutes before exposure to 10% NHS (lytic C dose) for 1 hour. Induced protection was seen with C8d pre-treatment, but was not altered by the presence of C8. *$p<0.01$, **$p<0.001$.

altered during lytic C attack on these cells. Cells were subject to lysis with NHS, with HI-NHS as control, and then stained for CD200, CD46 and CD59. Figure 4.15A shows that in the presence of 20% NHS, CD200 was lost from the cell surface (10 fold decrease, with majority of cells expressing no CD200). The identity of these cells was analysed using double staining with annexin-V-FITC. Figure 4.15B shows that using annexin-V-FITC/PI staining, 70-80% of cells were annexin+ /PI+, and that very few cells (0.5%) were annexin+/PI-. This indicated that cell death was necrotic, and that cells that were annexin+ were necrotic and not apoptotic. Double staining for CD200 and annexin-V-FITC showed that all annexin+ cells had low CD200 expression, confirming that dead cells had lost CD200. Analyses of CD46 and CD59 levels, alongside CD200, were performed using 10% NHS, and figure 4.16A shows that CD46 and CD59 were lost in a similar fashion to CD200, with 90% loss of CReg on PI+ cells. When analysed in triplicate, loss of CRegs and CD200 from lysed cells was statistically significant ($p<0.01$); expression of CReg and CD200 on cells surviving lytic C attack was reduced by 20%, suggesting that CReg and CD200 were shed from these cells, or that lysed cells were from the high expressing fraction of the population (figure 4.16B).

3.10. Sublytic C attack does not result in loss of CD200, CD46 or CD59

Since lytic attack resulted in loss of CD200, CD46 and CD59, even from surviving cells, the effect of prolonged sublytic C attack on these proteins was explored. For these experiments, C8d serum (5%) +/- C8 was used at sublytic concentrations. Cells were analysed after 1, 4 and 24h of incubation for levels of CD200, CD46 and CD59. No effect was seen at any time point for any of the proteins analysed, indicating that sublytic C attack did not alter the capacity of IMR-32 to defend against C attack via CRegs, or modulate microglial activation via CD200 (figure 4.17). This also confirmed that induced protection against lytic C attack seen with sublytic doses of C (figure 4.12 and figure 4.14) was not the result of alterations in CReg levels.

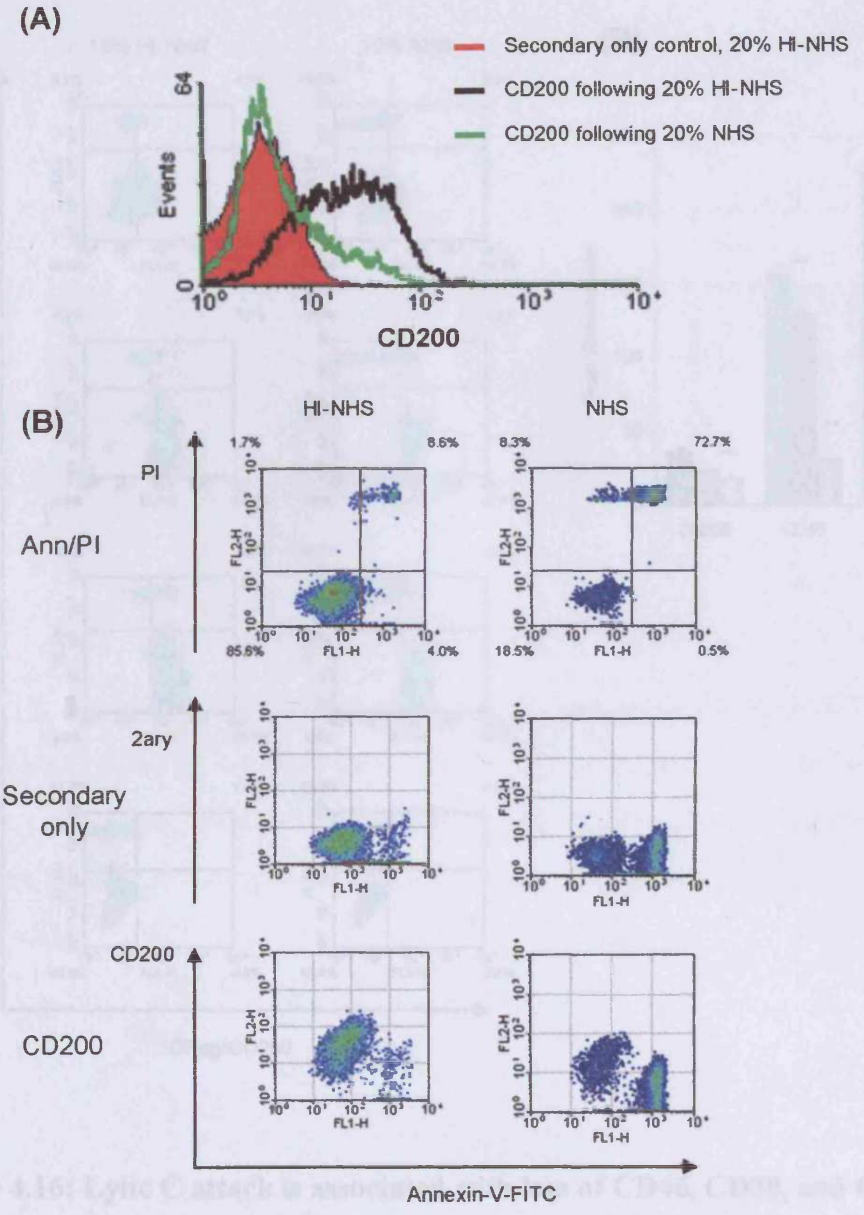


Figure 4.15: Lytic C attack is associated with loss of CD200. IMR-32 were harvested and attacked with a lytic dose of NHS, or HI-NHS as control, for 1hr, stained for CD200, and analysed by flow cytometry. **(A)** Lytic C attack caused a decrease in cell surface levels of the immune regulator CD200 on IMR-32. **(B)** Annexin/PI staining confirmed that cells were necrotic, since dead cells accumulated in the upper right quadrant, with very few cells in the lower right (early apoptotic) quadrant. Double staining confirmed that CD200 was decreased on annexin positive (ie dead) cells.

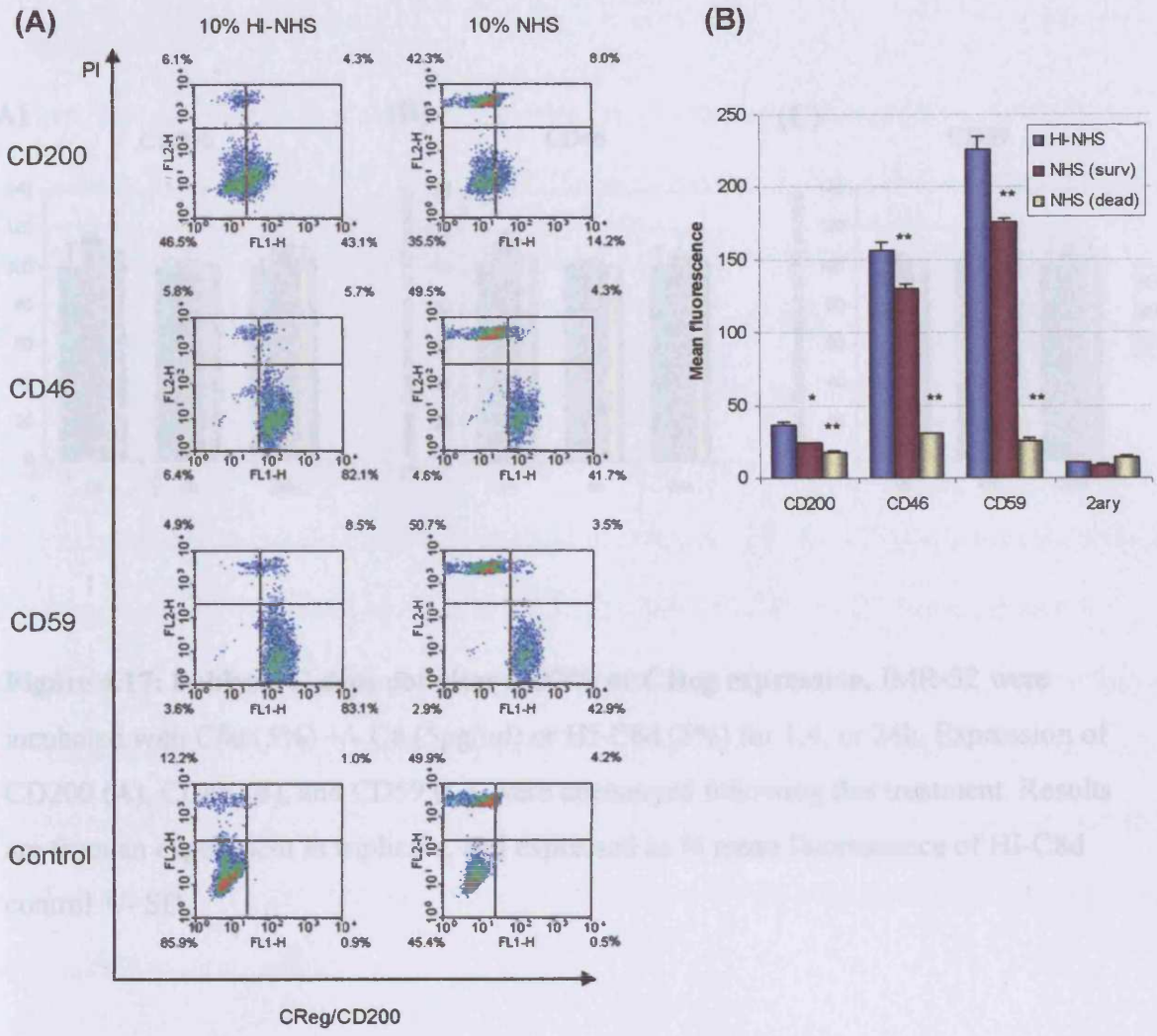


Figure 4.16: Lytic C attack is associated with loss of CD46, CD59, and CD200, from IMR-32. IMR-32 were harvested and attacked with NHS, or HI-NHS as control, at various doses, for 1hr, and then stained for CD200 or CReg. **(A)** Lytic C attack caused the loss of CD46 and CD59 from cells. All cells that had lost CReg were PI +ve, indicating that they had been lysed. No loss of CReg was observed from HI-NHS controls. **(B)** CReg and CD200 levels were analysed in triplicate. NHS caused a decrease in cell surface levels of the immune regulator CD200, and the CRegs CD46 and CD59. Results are expressed as MFI of CD200, CD46 or CD59. Results expressed as mean +/- SD. * $p<0.05$, ** $p<0.01$, each compared with relevant HI-NHS control.

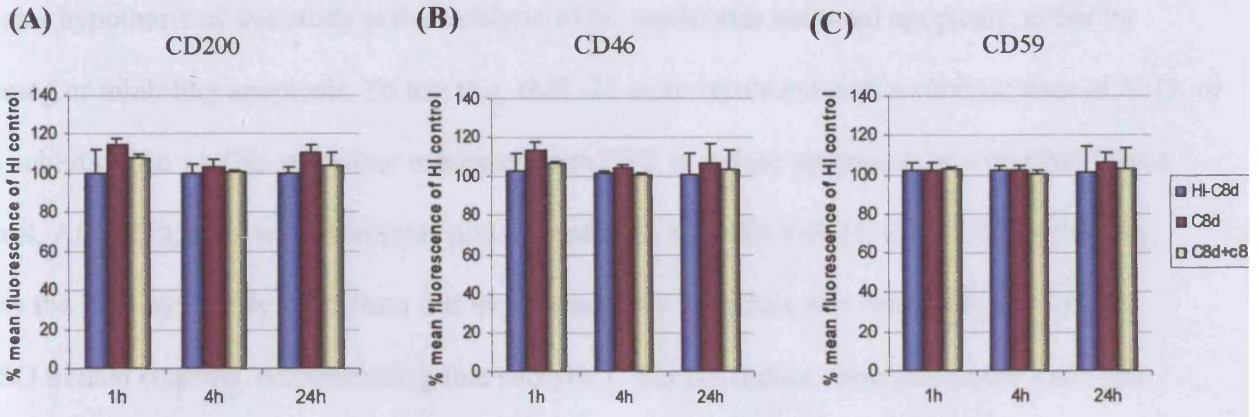


Figure 4.17: Sublytic C does not alter CD200 or CReg expression. IMR-32 were incubated with C8d (5%) +/- C8 (5µg/ml) or HI-C8d (5%) for 1,4, or 24h. Expression of CD200 **(A)**, CD46 **(B)**, and CD59 **(C)**, were unchanged following this treatment. Results are from an experiment in triplicate, and expressed as % mean fluorescence of HI-C8d control +/- SD.

3.11. Sublytic MAC attack does not result in modulation of CPT-induced IMR-32 apoptosis

A major hypothesis of this study is that sublytic MAC modulates neuronal apoptosis, either by inducing or inhibiting apoptosis. To test this, IMR-32 were incubated with a sublytic dose of NHS, or with sublytic C8d +/- C8, and either incubated with CPT to induce apoptosis, or with DMSO as a control. After 24h, cells were harvested and stained with annexin-V-FITC and PI. Figure 4.18A shows the flow cytometry plots from this experiment. No apoptosis was observed in any of the DMSO treated controls, demonstrating that sublytic C did not induce apoptosis in this cell type. Apoptosis was induced in the CPT treated control in the presence of 5% FCS (to control for protein-related effects), and this was attenuated in the NHS, HI-NHS and C8d +/- C8 treated groups, indicating that human serum contained factors that inhibited CPT-induced apoptosis, but that this was not a C-mediated effect. This was confirmed when the experiment was performed in triplicate with additional controls including HI-C8d and NHS at 2.5% (figure 4.18B). Apoptosis was inhibited in all cases, and no statistically significant differences were found between heat-inactivated serum groups and the C-sufficient serum groups.

Since the serum-related effects were very prominent in the inhibition of CPT-induced apoptosis, it was possible that they were masking subtle MAC-mediated effects. The experiment was therefore repeated using the reactive lysis system, using both purified components only, and formation of C5b-7 with C8d serum followed by addition of C8 and C9. Figure 14.19A and B shows that neither method of forming the MAC on the cell surface resulted in induction or inhibition of apoptosis. C5b-9 made using the C8d method appeared to show a small inhibition of apoptosis (5% reduction; figure 4.19A), but analysis of caspACE/PI stained samples and statistical analysis of multiple samples did not show a significant difference (figure 4.19B and figure 4.20).

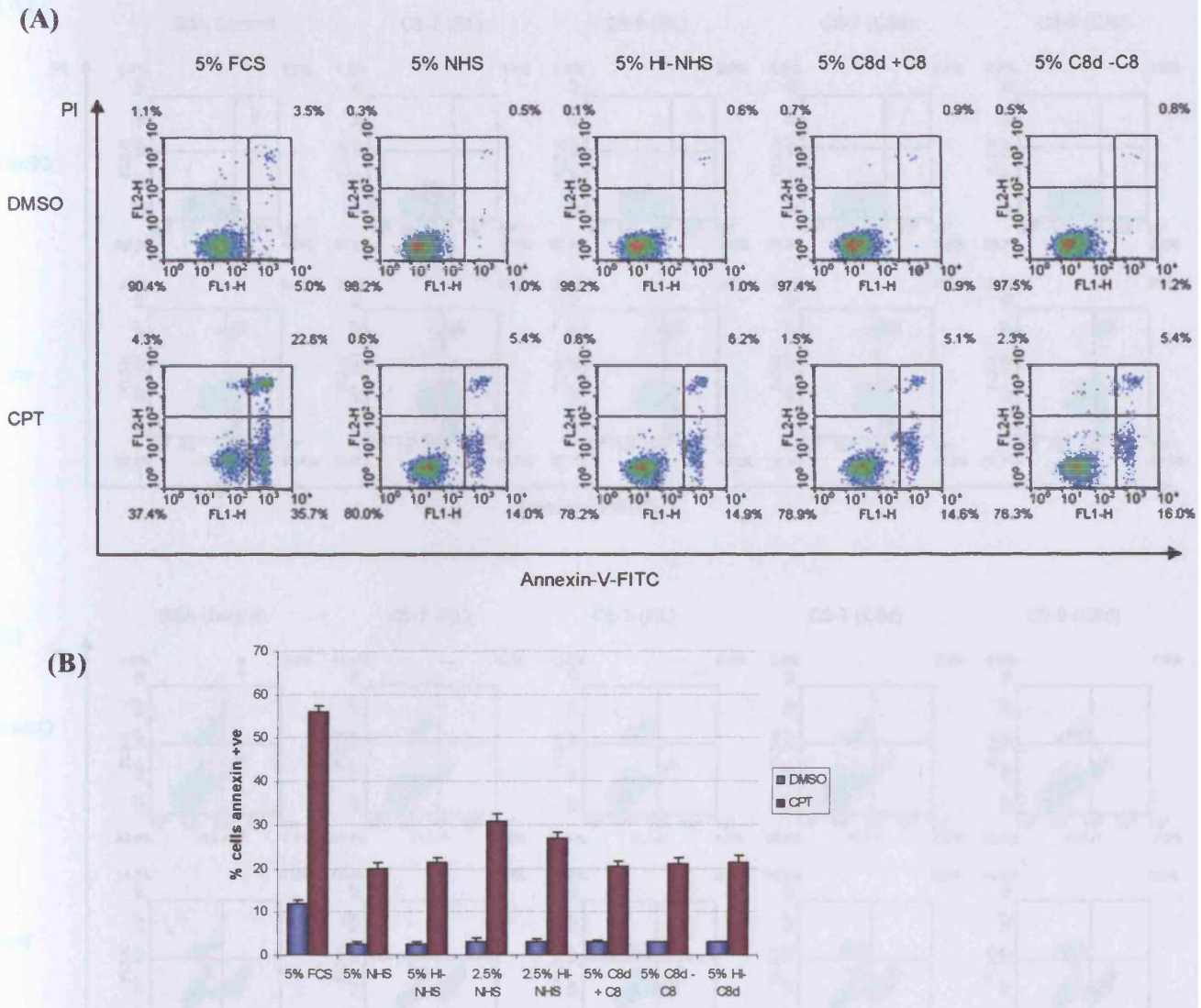


Figure 4.18: Sublytic C attack does not alter induction of IMR32 apoptosis. IMR-32 were incubated with CPT (1μM) to induce apoptosis, or DMSO as control, and either FCS (5%), NHS (5% or 2.5%), HI-NHS (5% or 2.5%), C8d (5%) +/- C8 (5μg/ml) or HI-C8d (5%) for 24h and then analysed by flow cytometry. **(A)** Flow cytometry annexin-V-FITC/PI plots show that sublytic C attack did not result in induction of apoptosis (DMSO controls) after 24h. CPT treatment resulted induction of apoptosis, and incubation with all variations of human serum resulted in inhibition of apoptosis. There was no difference between NHS and HI-NHS controls, or between C8d + C8 and C8d – C8. **(B)** Results were analysed in triplicate. There was no statistically significant difference in the number of annexin positive cells in CPT treated groups between NHS and HI-NHS controls, or between C8d + C8 and C8d – C8 or HI-C8d controls. Experiments were performed in triplicate, and results are expressed as % cells annexin positive +/- SD.

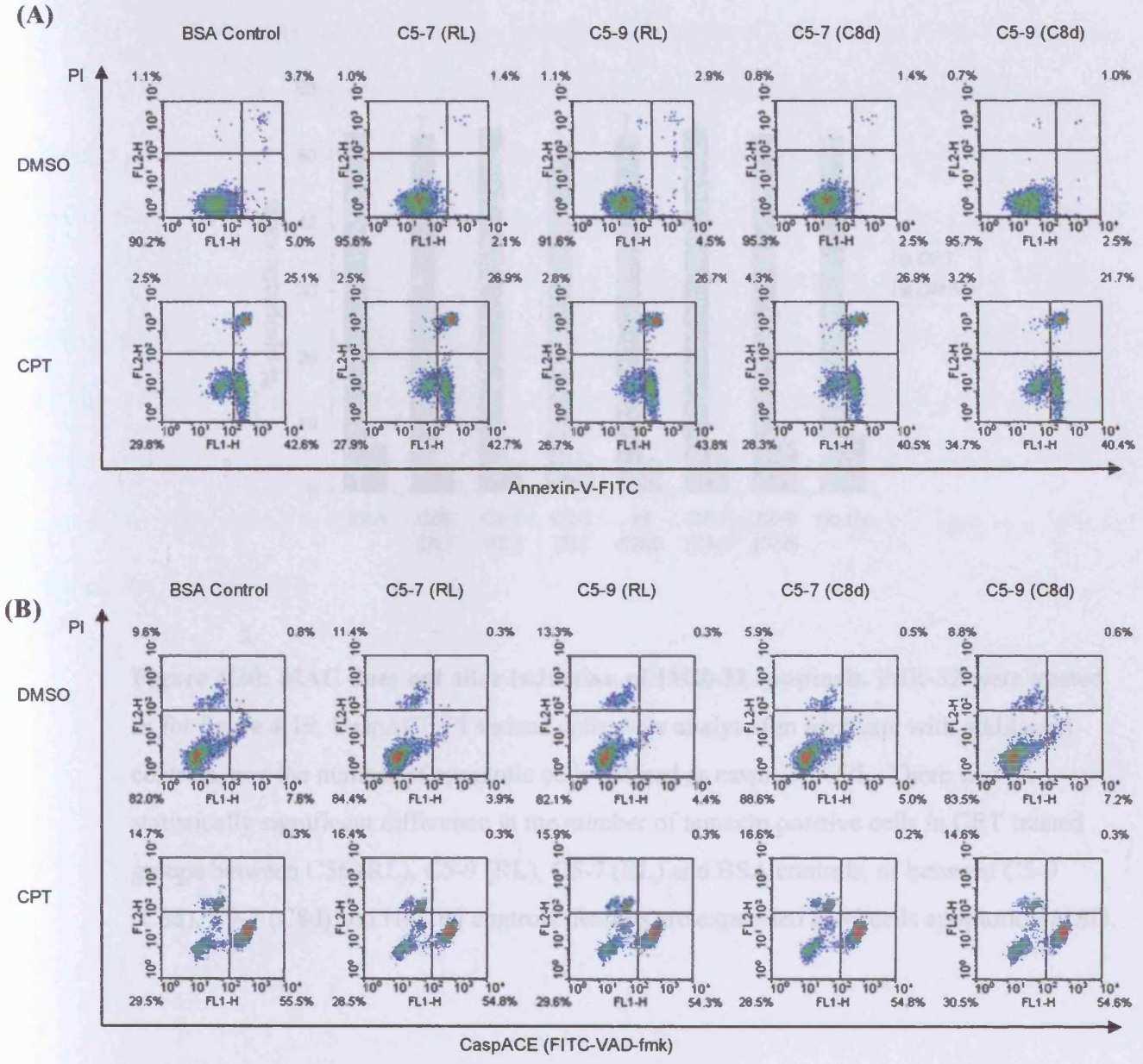


Figure 4.19: Sublytic MAC attack does not alter induction of IMR-32 apoptosis. IMR-32 were incubated with CPT (1μM) to induce apoptosis, or DMSO as control, with either BSA as protein control or C5-7 made using reactive lysis (RL) or by incubation with C8d serum (C8d), or with C5-9 made as for C5-7 +C8+C9. Cells were incubated for 24h and then analysed by flow cytometry. **(A)** Flow cytometry annexin-V-FITC/PI plots show that sublytic MAC attack did not result in induction of apoptosis (DMSO controls) after 24h. Neither C5-7 and C5-9 (RL or C8d) treatment altered numbers of apoptotic cells compared with BSA control. **(B)** Flow cytometry caspACE/PI plots confirmed the observations made with annexin-V-FITC/PI. MAC formed using RL of C8d did not induced or inhibit apoptosis

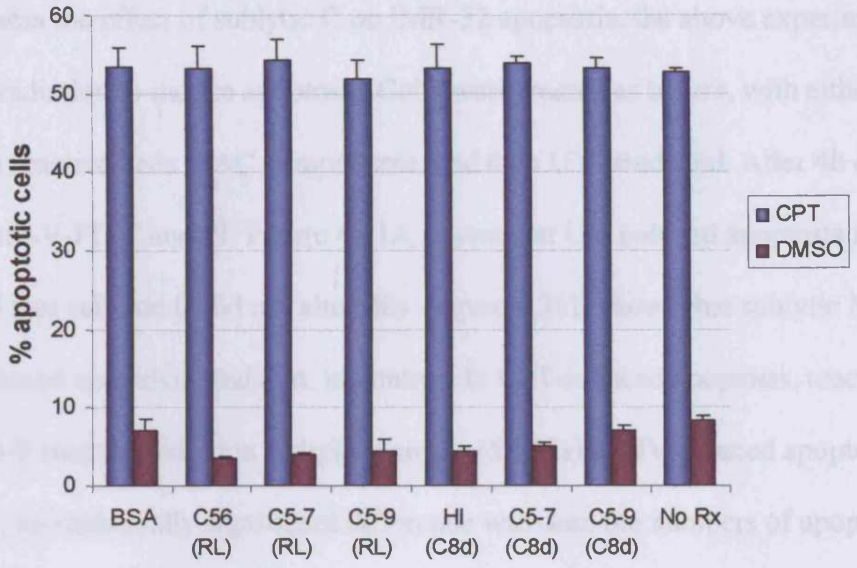


Figure 4.20: MAC does not alter induction of IMR-32 apoptosis. IMR-32 were treated as for figure 4.19. CaspACE/PI stained cells were analysed in triplicate with additional controls, and the number of apoptotic cells defined as caspACE+/PI-. There was no statistically significant difference in the number of annexin positive cells in CPT treated groups between C56 (RL), C5-9 (RL), C5-7 (RL) and BSA controls, or between C5-9 (C8d), C5-7 (C8d) and HI-C8d controls. Results are expressed as % cells apoptotic +/- SD.

3.12. Sublytic MAC attack does not result in modulation of UV-induced IMR-32 apoptosis

In order to further assess the effect of sublytic C on IMR-32 apoptosis, the above experiment was repeated using UV irradiation to induce apoptosis. Cells were treated as before, with either a sublytic dose of NHS, or with reactive lysis MAC components, and then UV-irradiated. After 4h cells were analysed using annexin-V-FITC and PI. Figure 4.21A shows that UV induced apoptosis in 80% of IMR-32 after 4h, and that sublytic C did not alter this. Figure 4.21B shows that sublytic MAC does not modulate UV-induced apoptosis, and that, in contrast to CPT-induced apoptosis, reactive lysis to both the C5-7 and C5-9 stages resulted in a slight increase (5-10%) in UV induced apoptosis. When analysed in triplicate, no statistically significant difference was seen the numbers of apoptotic cells in each group (figure 4.21C).

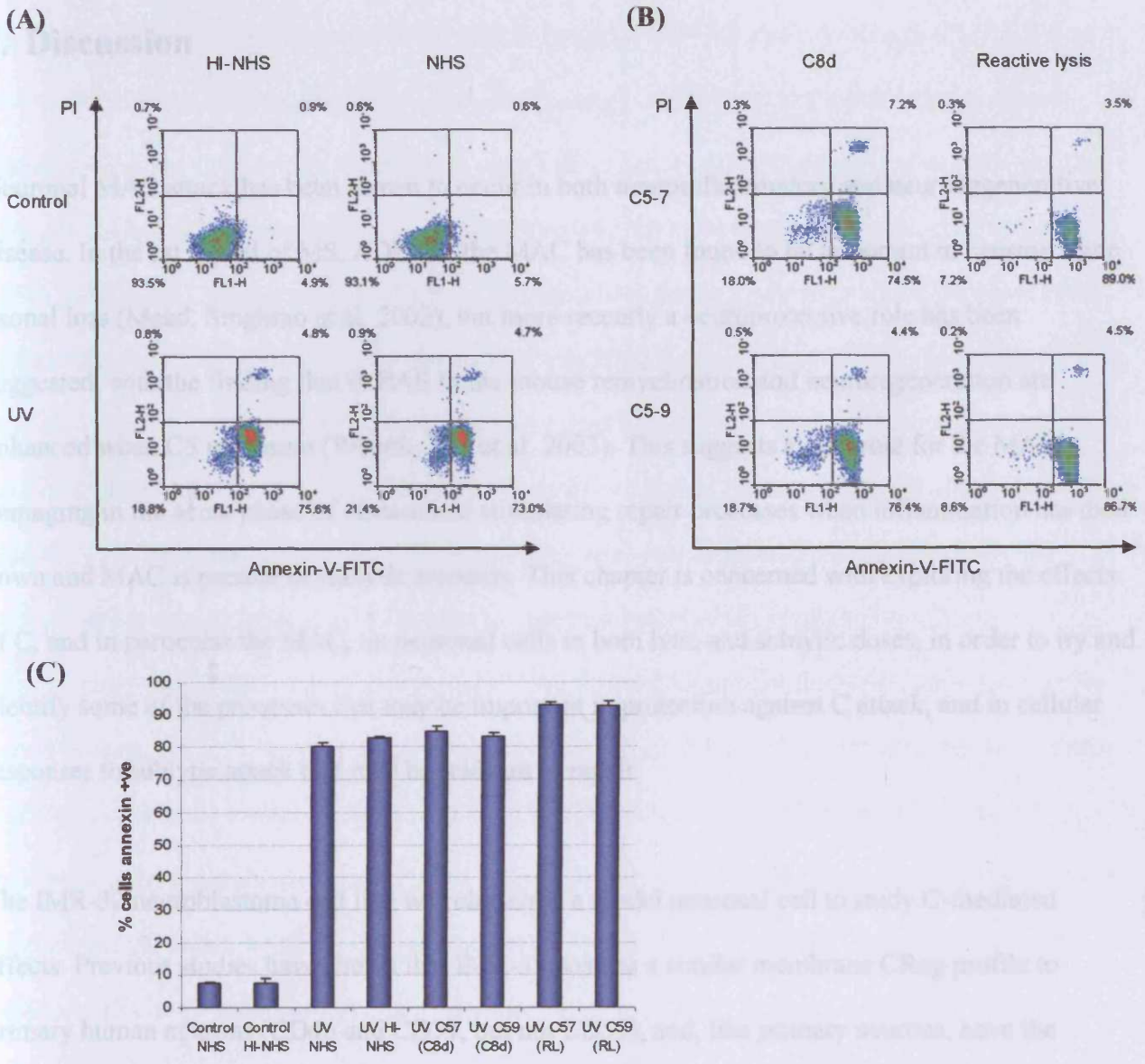


Figure 4.21: Sublytic MAC attack does not alter UV induction of IMR32 apoptosis. IMR-32 were treated with either 5% NHS or HI-NHS, or C5-7 (made using C8d or reactive lysis) +/- C8 and C9, and irradiated with UV. After 4h, cells were stained with annexin-V-FITC/PI and analysed by flow cytometry **(A)** Flow cytometry annexin-V-FITC/PI plots show that sublytic C attack did not result in induction of apoptosis (controls). Both NHS and HI-NHS treated cells had similar numbers of apoptotic cells after UV treatment. **(B)** Sublytic MAC, with C5-7 formed using either C8d or reactive lysis (RL), did not attenuate UV-induced apoptosis in IMT-32. **(C)** Results from all experiments were analysed in triplicate. There was no statistically significant difference in the number of annexin positive cells in UV treated groups between C5-9 (RL) and C5-7 (RL) controls, or between C5-9 (C8d) and C5-7 (C8d) controls. Results are expressed as % cells annexin positive +/- SD.

4. Discussion

Neuronal MAC attack has been shown to occur in both neuroinflammatory and neurodegenerative disease. In the rat model of MS, ADEAE, the MAC has been found to be important in causing acute axonal loss (Mead, Singhrao et al. 2002), but more recently a neuroprotective role has been suggested, with the finding that in EAE in the mouse remyelination and neuroregeneration are enhanced when C5 is present (Weerth, Rus et al. 2003). This suggests a dual role for the MAC: damaging in the acute phase of disease and stimulating repair processes when inflammation has died down and MAC is present in sublytic amounts. This chapter is concerned with exploring the effects of C, and in particular the MAC, on neuronal cells in both lytic and sublytic doses, in order to try and identify some of the processes that may be important in protection against C attack, and in cellular responses to sublytic attack that may be relevant to repair.

The IMR-32 neuroblastoma cell line was chosen as a model neuronal cell to study C-mediated effects. Previous studies have shown that IMR-32 possess a similar membrane CReg profile to primary human neurons (CD46 and CD59, but not CD55), and, like primary neurons, have the capacity to spontaneously activate C (Gasque, Thomas et al. 1996; Singhrao, Neal et al. 2000). In the first part of this study, it was confirmed that C is activated on IMR-32 cells in an antibody-independent fashion, that IMR-32 bind C1q directly in a dose-dependent fashion, and that activation of C on these cells is via the classical pathway. At present, the binding partner of C1q on neuronal cells is not known, and was not further investigated as part of this study.

Since the hypotheses of the chapter concerned the effects of the MAC on neuronal cells, a reactive lysis system was developed in order to study these effects in isolation from other C components and

serum factors. Purified components were used to build the MAC from C56 through C5-7, C5-8 and C5-9. C56 was made using a freeze-thaw method, using a modification of a published protocol (Dessauer, Rother et al. 1984). Haemolysis assays demonstrated that reactive lysis worked, but the reagents did not cause significant lysis of IMR-32 under the same conditions, even after blocking of CD59. Nucleated cells are intrinsically more resistant to C attack than erythrocytes (Morgan 1989), so this result was not surprising. The presence of the MAC on the surface of IMR-32 attacked using reactive lysis was confirmed using flow cytometry, and calcium flux assays demonstrated that reactive lysis MAC attack on IMR-32 resulted in calcium influx, the magnitude of which showed a dose-response relationship with the amount of C56 cells were incubated with. Importantly, the calcium influx was reversible, and returned to near baseline after 10 minutes. This clearly demonstrated that IMR-32 were actively defending themselves against the MAC, since in order for this to happen, the MAC pore must be closed or removed from the cell membrane, and the cell must activate mechanisms to counteract the ion flux. The importance of this was underlined by the observation, using single cell imaging, that cells undergoing lytic C attack lost the ability to control intracellular calcium levels, and calcium concentration rose sharply to a maximum, and after about 5 minutes, cells lost membrane integrity and died.

In the previous chapter, it was demonstrated that neuronal cells in early apoptosis are more susceptible to C-mediated killing, a phenomenon that is, at least partially, due to increased C-activation as a result of CD46 loss. It has recently been shown that caspases cleave plasma membrane calcium pumps in apoptotic neurons, rendering these cells inefficient at handling calcium flux changes (Schwab, Guerini et al. 2002). It is therefore possible that a further reason why apoptotic neurons are susceptible to C-mediated killing is that they cannot handle the calcium influx through the MAC pore. This was investigated using the reactive lysis calcium flux assay, and the results showed that apoptotic cells responded to sublytic MAC attack in an abnormal fashion, with a higher

peak intracellular calcium concentration, and a longer and incomplete recovery compared to normal controls. This implied that calcium handling was impaired in these cells, and may contribute to the increase in C-mediated killing in apoptotic neurons.

The calcium flux data also demonstrated that the kinetics of MAC attack were different in serum-based lytic C attack compared with the sublytic reactive lysis system. Lytic C attack using serum typically induced a slower calcium response than that produced by sublytic reactive lysis, and cells did not show clear phases of calcium influx and recovery. Instead, once calcium levels had increased to a high level in serum-attacked cells, the cells died. This difference is likely to be due to progressive and cumulative C activation on cells with serum-based attack, where the number of MAC pores increase with time on the cell surface. Initially the cell can respond and cope, but eventually the recovery mechanisms are overwhelmed, and calcium levels rise dramatically. No recovery from this situation is possible, as more MAC pores are continually being deposited than can be removed by the cell. In reactive lysis, the cell receives a one-off MAC attack, as all C5-7 sites are formed at the same time, and C8 and C9 are then added together. The rate limiting factor for calcium flux changes is the time taken for the C8 and C9 to insert into the membrane on a C5-7 complex, not in the time it takes to build up a given number of MAC pores. If the cell can cope with this single all-out attack, then it will survive and recover, as no further MAC pores are being formed. Thus reactive lysis does not model the physiological C activation on cells accurately, but does isolate MAC effects from other C effects. In contrast, serum-based MAC attack using C8d serum with or without C8 reconstitution is more physiologically relevant, but still suffers from the presence of serum-related confounding factors.

One well-known sublytic effect of the MAC is the phenomenon of induced protection (Morgan 1989;

Reiter, Ciobotariu et al. 1992; Jurianz, Ziegler et al. 1999). When cells are exposed to sublytic doses of the MAC, they become more resistant to subsequent attack by lytic doses of C. This phenomenon also occurs with other pore-formers, and indeed sublytic attack by one pore-former can induce cross-protection to another (Reiter, Ciobotariu et al. 1995). Calcium has been implicated in the mechanism of induced protection (Reiter, Ciobotariu et al. 1995), and active RNA and protein synthesis must also occur for the effect to be seen (Reiter, Ciobotariu et al. 1992). A number of studies have examined the mechanisms involved, and have shown that protein kinase C and ERK signalling are involved in mediating the effect in leukaemia cell lines (Kraus and Fishelson 2000; Kraus, Seger et al. 2001), and heat shock proteins also play a role (Fishelson, Hochman et al. 2001). One group has shown that a large C-induced protein (L-CIP) is synthesised in response to sublytic attack (Reiter and Fishelson 1992), although its precise role has yet to be defined. I have shown that neuronal cells also display this phenomenon, indicating that the brain is well equipped with intrinsic mechanisms to resist lysis. The effect observed was dose-dependent, and also occurred when C8d serum was used, suggesting that MAC was either not involved or not essential for the process. When the phenomenon was investigated using reactive lysis, no protection was seen, which suggested that this hypothesis was correct. It is possible that other C activation products signal to the cell, perhaps via a CReg on the cell membrane. In IMR-32, CD46 would be one such candidate since signalling via this molecule has been shown in other systems (Kurita-Taniguchi, Fukui et al. 2000), although it is also possible that C1q may be responsible as it too could signal to the cell through specific receptors (Leigh, Ghebrehiwet et al. 1998), or that another heat-labile serum factor is important. The precise mechanism has yet to be defined in neuronal cells, but in any case exposure to low doses of C does induce protection to the lytic effects of C, and this may help to preserve neuronal integrity under inflammatory conditions.

The next set of experiments examined the effect of lytic and sublytic C attack on the CRegs CD46

and CD59, and on the immune modulator CD200. Apoptosis resulted in the loss of CRegs from the neuronal cell surface (Chapter 3). This part of the study revealed that this was not limited to programmed cell death, but also occurred as part of C-mediated necrosis of cells. The pattern of loss was different, however, with CD46 and CD59 expression reduced to a similar degree over the same short time frame, in contrast to their different patterns during apoptosis with CD46 loss occurring much earlier than CD59, and to a greater extent. This suggested that the mechanism of CReg loss was the same during lysis, possibly as a result of non-specific vesiculation or fragmentation of the cell. Sublytic C attack, in contrast, did not alter levels of CRegs on IMR-32, despite the observation that cells surviving lytic doses of NHS showed reduced expression of CRegs. This indicates that chronic low-grade C activation does not induce further protection mechanisms relating to membrane-bound CReg, and that other factors related to local necrotic cell death may also be relevant in CReg loss. These data also confirmed that induced protection to C-mediated killing by sublytic C was not due to changes in CReg.

CD200 also followed the same pattern of loss during lytic C attack as CD46 and CD59. CD200 is expressed on a variety of cells types, including neuronal cells, and has a role in dampening microglial activation in the brain (Hoek, Ruuls et al. 2000; Wright, Jones et al. 2001). In the context of lysis, loss of CD200 may play a role in activating microglia and promoting inflammation, although the presence of other necrotic debris may render this effect superfluous. No effect was seen on CD200 levels following sublytic C attack, suggesting that sublytic C does not have a significant role in modulating immune responses to neuronal cells.

The final set of experiments examined the role of C, and in particular the MAC, in modulating neuronal apoptosis, since a number of studies have shown MAC-mediated inhibitory effects on apoptosis in the CNS (Soane, Rus et al. 1999; Soane, Cho et al. 2001; Niculescu, Weerth et al. 2004).

Using serum-based approaches and reactive lysis with purified terminal pathway components, no effect of sublytic MAC attack was seen on IMR-32 apoptosis induced using CPT or UV irradiation. This suggests that while some cells in the CNS, such as oligodendrocytes, respond to sublytic MAC in a way that promotes survival in the face of apoptotic stimuli, other cells such as neurons do not. It is possible that the methods used to induce neuronal apoptosis in this study are not sensitive to modulation by MAC, since different methods were used to induce oligodendrocyte apoptosis, ie incubating with TNF-α or by inducing differentiation (Soane, Rus et al. 1999). However, MAC-mediated inhibition of oligodendrocyte apoptosis in this study was a result of a reduction in caspase-3 activation, which is also important in CPT-induced IMR-32 apoptosis (see chapter 3). Thus the lack of protection noted is unlikely to be solely the result of using different methods of apoptosis induction.

This chapter has demonstrated a number of features of lytic and sublytic C attack on IMR-32 cells. These cells displayed recovery from MAC-mediated attack by removing lytic lesions and normalising intracellular calcium levels following insertion of the MAC pore, a phenomenon that was impaired in apoptotic cells. Sublytic C also caused induced protection to further lytic C attack, but had no effect on induction of apoptosis by UV or CPT. Finally, lytic C attack caused the loss of CD200, CD46 and CD59 from the cell surface, but sublytic C did not. Thus, in terms of the hypotheses set out in the introduction, neuronal cells do possess mechanisms to defend themselves against C attack, independent of classical CReg; and sublytic C attack does protect against cell death, but only against C-mediated lysis, and not against CPT or UV-induced apoptosis. Much of the data presented here has yet to be fully investigated: the mechanism by which neuronal cells restore normal intracellular calcium levels following MAC attack has yet to defined, as has the basis for its impairment in apoptotic cells. Induced protection in these cells appears to follow a largely MAC-independent pathway, unlike results in other studies, which also remains to be further explored. Thus neuronal

cells possess a set of defence mechanisms against C attack in common with many other cell types, but do not express sublytic MAC-mediated apoptosis modulation mechanisms. The latter are likely to be highly variable between different cell types, since both apoptosis induction and inhibition of apoptosis have been previously described, and in some cell types, such as IMR-32, no effect is seen.

Chapter 5: Complement and neuronal apoptosis – interaction *in vivo*

1. Introduction

Neuronal death occurs via a number of mechanisms under pathological conditions in vivo (Fawcett 2001). These include attack by the C system, which classically causes necrosis of neurons; direct toxic effects of pathogens or toxic compounds; metabolic and oxidative stress; and excitotoxicity. Excitotoxic cell death occurs when compounds such as the neurotransmitter glutamate and its agonists cause excessive stimulation of the neuron. This leads to a large influx of calcium that initiates a cascade of intracellular events that eventually leads to death by apoptosis or necrosis. Excitotoxic neuronal death has been shown to occur in both neuroinflammatory and neurodegenerative disease processes (Pitt, Werner et al. 2000; Hynd, Scott et al. 2004; Mishizen-Eberz, Rissman et al. 2004).

Kainic acid is a potent agonist of kainate glutamate receptors and is commonly used as a model of excitotoxic neuronal death in vivo (Lothman and Collins 1981; Sperk, Lassmann et al. 1985; Ino and Chiba 2001; Gilliams-Francis, Quaye et al. 2003). After intraperitoneal injection in rats, animals display a series of characteristic behavioural changes. Initially animals become motionless and stare; following this after 30 minutes they display "wet dog shakes". At higher doses (7-10mg/kg) they start to develop mild limbic convulsions, and then more extensive seizures, involving rearing and falling over (Sperk, Lassmann et al. 1985). The development of neuronal pathology appears to be dependent on seizure activity, with only minor changes seen if animals remain seizure-free or receive diazepam

to terminate convulsions (Pollard, Charriaut-Marlangue et al. 1994).

Kainic acid treatment induces neuronal death in the hippocampus, particularly in the sensitive CA1 and CA3 areas (Lothman and Collins 1981; Sperk, Lassmann et al. 1985). The entorhinal cortex is also involved early, and with increasing doses damage spreads to involve the dentate gyrus and other areas of the cortex and striatum (Lothman and Collins 1981). This pattern of pathology is dose-dependent and follows behavioural and electroencephalographic changes in the animals. Neuronal death under these conditions has been shown to involve cellular shrinkage and caspase-3 activation, with subsequent PARP cleavage and TUNEL positivity, suggesting that cell death is apoptotic in nature (Gilliams-Francis, Quaye et al. 2003). Others have disputed this, claiming that the cell death seen is more characteristic of necrosis (Fujikawa, Shinmei et al. 2000; Puig and Ferrer 2002). This may be a result of strain differences and/or different routes of administration of agent.

A small number of studies have examined the role of C in kainic acid treated animals. C1q has been shown to be induced after kainic acid administration (Goldsmith, Wals et al. 1997), and C5aR mRNA up-regulation has been reported (Osaka, McGinty et al. 1999). The only CReg so far shown to be altered is clusterin, which is induced in areas of neuronal damage (Rozovsky, Morgan et al. 1994). Importantly for this study, C5 deficient mice have been shown to develop more extensive neuronal loss following kainic acid administration than their C5 sufficient counterparts, suggesting that either the MAC or C5a are neuroprotective (Pasinetti, Tocco et al. 1996). Astrocytes from C5 deficient animals also had heightened responses to inflammatory stimuli such as LPS, and neuronal firing was depressed (Pasinetti, Tocco et al. 1996). Thus far, it is C5a that has been implicated in this phenomenon of neuroprotection, as co-infusion of C5a with kainic acid reduced neuronal damage, and in vitro studies of signalling pathways suggested that suppression of caspase-3 activity by C5a

prevented apoptosis of murine neurons (Osaka, Mukherjee et al. 1999). However, a role for the MAC has not been investigated in this setting, and could be important as studies in EAE have shown a protective role for the MAC, particularly in the neuroregenerative/ chronic phase of the disease process (Weerth, Rus et al. 2003). Alternatively, MAC may be damaging in an acute excitotoxic context, since it has been shown to induce seizures when it is formed by the sequential infusion of C5b6, C7, C8 and C9 into the CNS in the rat (Xiong, Qian et al. 2003).

Kainic acid-induced excitotoxic neuronal death was deemed a good model to study the interaction of MAC, CReg and C activation with apoptotic cells in vivo. It targets a highly specific subset of neurons, is well characterised behaviourally and neuropathologically, and has been shown to involve C in its pathogenesis. In particular, it is possible to analyse the specific role of the MAC as C6 deficient animals are available in-house.

The hypotheses are:

1. **The MAC is neuroprotective under excitotoxic conditions in vivo**
2. **Apoptotic neurons lose CReg in vivo and have increased deposition of C activation products**

The study was carried out using intraperitoneal (IP) kainic acid injection in rats, and harvesting of the brain for cryosections. Immunohistochemistry and fluorescence microscopy were then used to examine the brain for the presence of apoptotic neurons, CReg and C activation products.

2. Methods

2.1. Disease induction and clinical scoring

Lewis rats deficient in C6 ($C6^{-/-}$) were used for the study in accordance with Home Office and local guidelines. Kainic acid (7mg/kg) was administered by IP injection, and rats were either reconstituted with human C6 (8mg/kg; 5mg/ml) or received a control injection (normal saline) by IP injection. Animals were observed for the next 5 hours, and clinical scores recorded every hour. Clinical scoring was as follows:

0 – normal behaviour

1 – staring and immobility

2 – wet dog shakes

3 – mild limbic convulsions (head bobbing, twitching of vibrissae, yawning and other automatisms)

4 – convulsions (limb twitching, rearing and falling backwards, generalised tonic-clonic seizures)

5 – death.

After 24 hours, all animals were sacrificed by IP injection of sodium pentobarbital and cervical dislocation.

2.2. Tissue processing

Blood was harvested by cardiac puncture, allowed to clot, and spun at 1000g for 15 minutes to separate serum. Serum samples were kept frozen at -80°C until analysis. Brains were dissected and

snap frozen in isopentane at -40°C, before storage at -80°C until analysis. Brain hemispheres were sectioned coronally using a cryotome at -16°C, transferred to slides pre-treated for electrostatic adherence, dried overnight at 22°C and stored at -80°C until analysis.

2.3. Staining of tissue sections

2.3.1. Immunohistochemistry

Tissue sections were dried at 22°C and fixed in acetone for 1 minute. For CD59 and CD55 staining sections were fixed in 100% methanol for 1 minute. Slides were allowed to dry, and were washed in PBS for 5 minutes before blocking with PBS/ 1% BSA for 1 hour at 22°C. Slides were then incubated with primary antibodies (10μg/ml) diluted in PBS/BSA overnight at 4°C in a humidity chamber. Slides were then washed 3 times in PBS for 5 minutes at 22°C, and incubated with the fluorochrome conjugated secondary antibody (goat anti-mouse FITC 1/50 or donkey anti-mouse rhodamine 1/200) and DAPI (1/2000) in PBS/BSA for 1 hour at 22°C in the dark. Slides were then washed 3 times in PBS for 5 minutes at 22°C, the excess moisture carefully wiped clear, and mounted using Vectashield mounting medium and coverslip. Slides were analysed on a fluorescence microscope.

2.3.2. Apoptosis detection

For caspACE staining, slides were first dried at 22°C and fixed in acetone for 1 minute. Slides were allowed to dry, and were washed in PBS for 5 minutes before incubating with 5μM caspACE in PBS/ BSA for 30 minutes at 22°C in the dark. Slides were then washed 3 times in PBS for 5 minutes at 22°C, the excess moisture carefully wiped clear, and mounted using Vectashield mounting medium and coverslip. Slides were viewed on a fluorescence microscope and analysed using Openlab software (Improvision, Coventry, UK).

3. Results

3.1. Purification of human C6

In order to assess the effect of the MAC on neuronal apoptosis in vivo, it was decided to use $C6^{-/-}$ Lewis rats reconstituted with human C6 or carrier control in a kainic acid model of neuronal degeneration. Human C6 was purified from human plasma using an anti-C6 mAb column, as described in Materials and Methods. Figure 5.1A shows that two bands were seen on Coomassie staining of an SDS-PAGE gel. C6 was likely the lower of the two bands (Mr 114kDa). The upper band was likely either contamination with antibody from the column, or C5. The latter is possible, since earlier Western blots on C6 preparations with anti-C5 antibody had detected a band at this weight (Chapter 4, figure 4.4B). The identity of the C6 band was confirmed by Western blotting (figure 5.1B). An attempt to remove the contaminant was made using gel filtration chromatography (figure 5.1C). Two clear peaks were seen at 280nm, suggesting that the contaminant had been separated from the C6, but when a SDS-PAGE gel was run the band at 190-200kDa remained in the same fraction as the C6, and the contaminant removed by this procedure was a high Mr protein or aggregate that did not enter the gel (figure 5.1D). It was decided not to purify any further, since this contaminant was unlikely to cause significant problems in the study as the time course was short (24h).

3.2. Reconstitution of C6 deficient rat serum with human C6

Before using human C6 to reconstitute $C6^{-/-}$ rats in vivo, it was necessary to check that human C6 could reconstitute haemolytic activity in $C6^{-/-}$ rat serum in vitro. Figure 5.2 shows that $C6^{-/-}$ rat serum had no haemolytic activity. The small amount of colour that was seen was due to haemolysis caused during harvesting the blood, and was already present in serum prior to the assay. Human C6

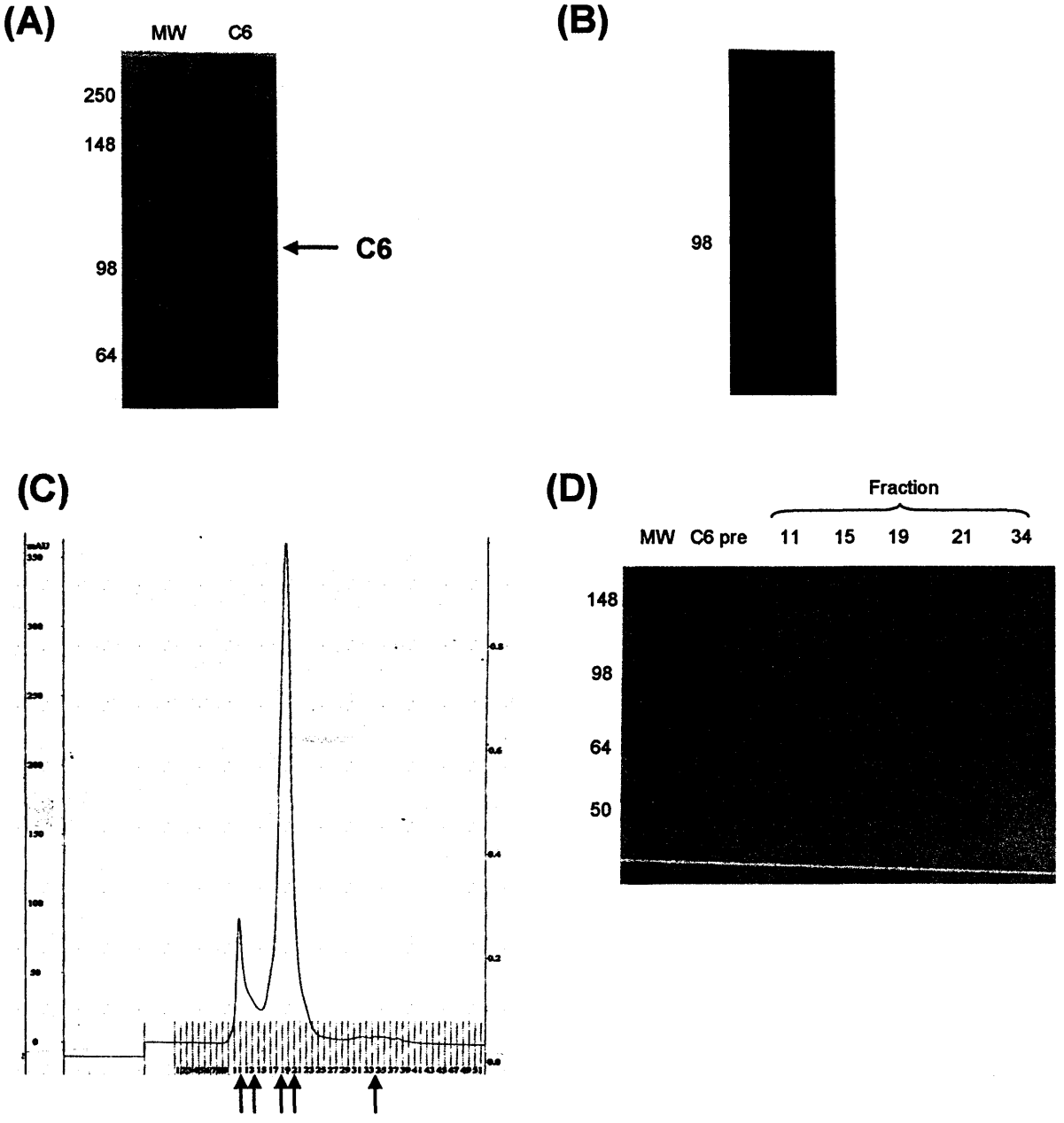


Figure 5.1: Purification of human C6. C6 was purified by affinity chromatography using anti-C6 mAb column. **(A)** Coomassie stained SDS-PAGE gel showed a major band of the correct MW for C6 (114kDa). A second, higher MW band was also seen (MW 160kDa), and a small band at the top of the gel. **(B)** Western blotting using anti-C6 mAb confirmed that the major band on Coomassie staining was C6. A strong lower MW band was also seen, most likely a C6 breakdown product. **(C)** Gel filtration 280nm absorbance profile showed separation of the C6 preparation into two peaks, but on analysis by SDS-PAGE **(D)** this had not separated C6 from the major 160kDa contaminant. Arrows in (C) indicate fractions analysed in (D).

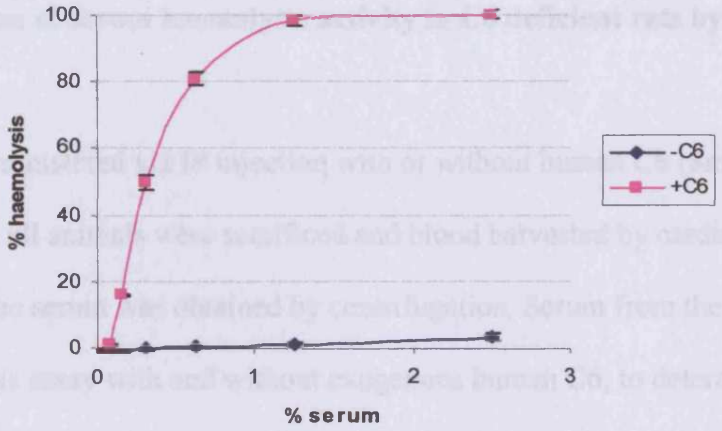


Figure 5.2: Reconstitution of $C6^{-/-}$ rat serum with human C6. To test the ability of the purified human C6 to reconstitute rat serum, human C6 (2μg/ml) was added to varying concentrations of C6 deficient rat serum. Good reconstitution was seen. A small amount of colour was present at the highest concentrations of C6 deficient rat serum alone, reflecting haemolysis occurring during harvesting of serum prior to the assay.

reconstituted $C6^{-/-}$ rat serum in vitro, indicating that reconstitution in vivo would work (figure 5.2).

3.3. Reconstitution of serum haemolytic activity in C6 deficient rats by adding back human C6

KA (7mg/kg) was administered via IP injection with or without human C6 (8mg/kg). At the end of the experiment (24h), all animals were sacrificed and blood harvested by cardiac puncture. Blood was allowed to clot, and the serum was obtained by centrifugation. Serum from the animals was then subject to a haemolysis assay with and without exogenous human C6, to determine the extent of the reconstitution of C6 activity. Figure 5.3A shows examples of haemolysis assays from two representative animals from each group. Animals that had not received human C6 in vivo had minimal haemolysis, but regained near-full haemolytic activity when exogenous human C6 was added back in vitro. Animals that had received human C6 in vivo demonstrated reconstitution of haemolytic activity at 24h. Figure 5.3B shows mean haemolysis curves for all animals in each group, and confirmed that human C6 reconstituted haemolytic activity in $C6^{-/-}$ rats. Both groups had similar maximal haemolytic activity when exogenous human C6 was added back. CH50 values were calculated for each group, and showed that the $C6^{-/-}$ + human C6 reconstituted to 50% haemolytic activity of the maximum after 24h (figure 5.4). These data indicated that IP human C6 effectively restored haemolytic activity in $C6^{-/-}$ animals.

3.4. KA induces seizures in both $C6^{-/-}$ rats and $C6^{-/-}$ rats reconstituted with C6

Following KA injection all animals displayed behavioural changes to varying degrees. The first change to be noted was episodes of staring and immobility lasting from a few seconds up to 3 minutes, and occurred 2-5 minutes following KA. Following this, after 30-60 minutes, animals displayed "wet dog shakes", and went on to mild limbic seizures, including chewing, yawning, head bobbing and twitching of vibrissae. These were prolonged, repetitive, and resistant to external stimuli.

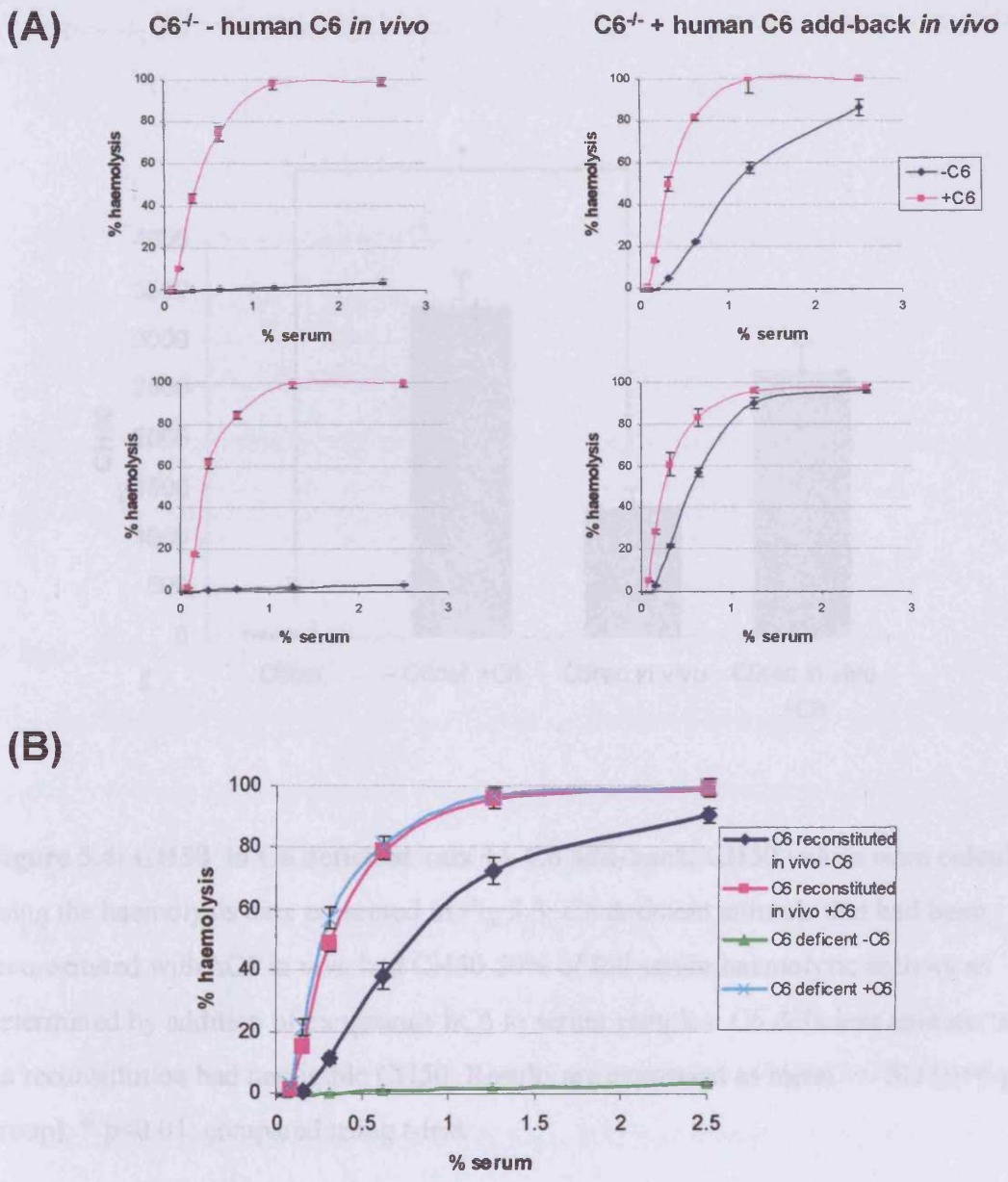


Figure 5.3: Haemolysis in C6 deficient rats +/- C6 add-back. C6 deficient rats were reconstituted with 8mg/kg human C6, or received PBS control. On testing the serum, full serum haemolytic activity was obtained by adding exogenous human C6 to the rat serum prior to the assay. **(A)** Haemolysis assays from two control animals (left panel) and two reconstituted animals (right panels). Haemolysis was only seen in the human C6 reconstituted animals, but this was not full reconstitution of haemolytic activity. **(B)** Haemolysis curves for all animals in the study. Reconstitution of haemolytic activity was seen in all C6 deficient animals that had received human C6. Results are expressed as mean haemolysis +/- SEM (n=6 per group).

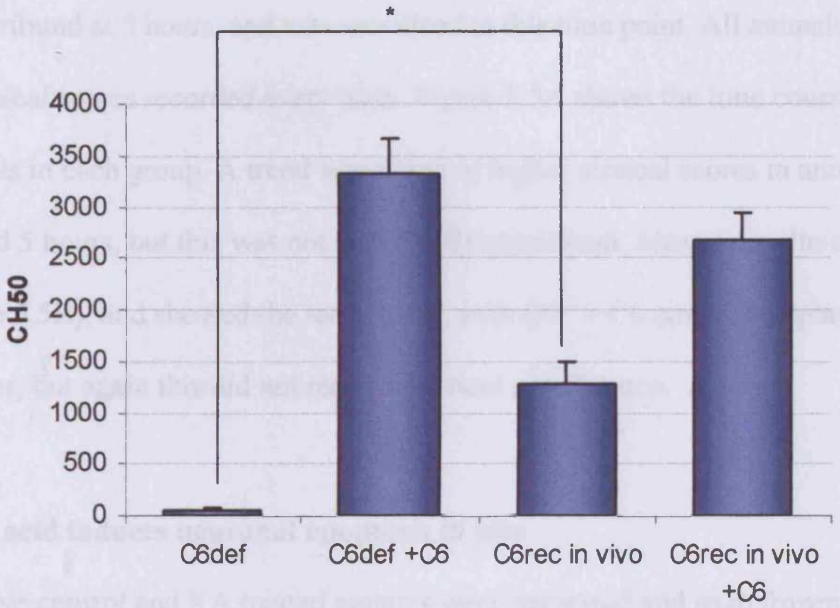


Figure 5.4: CH50 in C6 deficient rats +/- C6 add-back. CH50 values were calculated using the haemolysis data presented in Fig 5.3. C6 deficient animals that had been reconstituted with hC6 in vivo had CH50 50% of full serum haemolytic activity as determined by addition of exogenous hC6 to serum samples. C6 deficient animals with no reconstitution had negligible CH50. Results are expressed as mean +/- SD (n=6 per group). * $p<0.01$, compared using t-test.

Generalised seizures occurred in some animals after 1-2 hours, and persisted intermittently for 2-3 hours, and involved limb twitching, rearing, and falling backwards. One animal had severe seizures and became moribund at 5 hours, and was sacrificed at this time point. All animals were observed for 5 hours and clinical scores recorded every hour. Figure 5.5A shows the time course of the clinical scores of animals in each group. A trend was noted of higher clinical scores in animals reconstituted with C6 at 4 and 5 hours, but this was not statistically significant. Maximum clinical scores were analysed (figure 5.5B), and showed the same trend, with $C6^{-/-}$ + C6 animals displaying higher maximum scores, but again this did not reach statistical significance.

3.5. Kainic acid induces neuronal apoptosis *in vivo*

Brains from naïve control and KA treated animals were harvested and snap frozen in isopentane, and sectioned on a cryotome. After fixation, sections were stained with the caspACE (FITC-VAD-fmk) marker that stains activated caspases and therefore identifies apoptotic cells. Figure 5.6B shows that caspACE stained CA1 region hippocampal cells in KA treated animals, but did not stain cells in the naïve animal brain (figure 5.6A), suggesting that apoptosis was occurring in the brains of KA treated animals, but not controls. CaspACE positive cells were also diffusely present in the cortex and striatum of kainic acid treated animals (figure 5.6D and 5.6F respectively), but not controls (figure 5.6C and 5.6E). High power views of these cells demonstrated caspACE staining of axonal and dendritic extensions, indicating that they were neurons (figure 5.7). In order to confirm that these cells were indeed apoptotic, sections were also stained with an antibody to ssDNA that is specific for apoptosis. Double staining with caspACE demonstrated that both markers co-localised in the hippocampus of treated animals (figure 5.8), confirming that caspACE positive cells were apoptotic.

3.6. Promotion of excitotoxic neuronal cell death by MAC

Tissue sections from KA treated $C6^{-/-}$ and $C6^{-/-}$ + human C6 treated animals were stained for caspACE

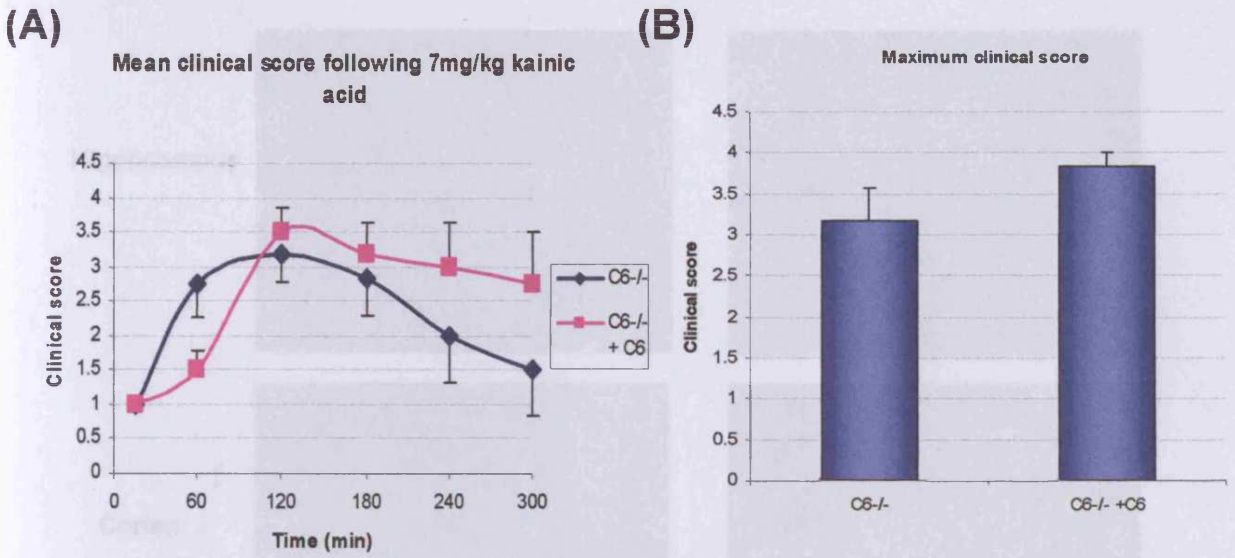


Figure 5.5: Clinical scores following administration of 7mg/kg kainic acid. (A) Mean clinical score following KA. All animals developed behavioural changes, but C6 deficient animals showed a trend towards lower scores than the human C6 reconstituted group at 4 and 5 hours. This did not reach statistical significance ($p=0.2$ at 5 hours, $n=6$). **(B)** Maximum clinical score also showed a trend towards higher scores with human C6 reconstituted animals, but this also did not reach statistical significance ($n=6$). Mann-Whitney U test used for statistical analysis. Results expressed as mean +/- SEM.

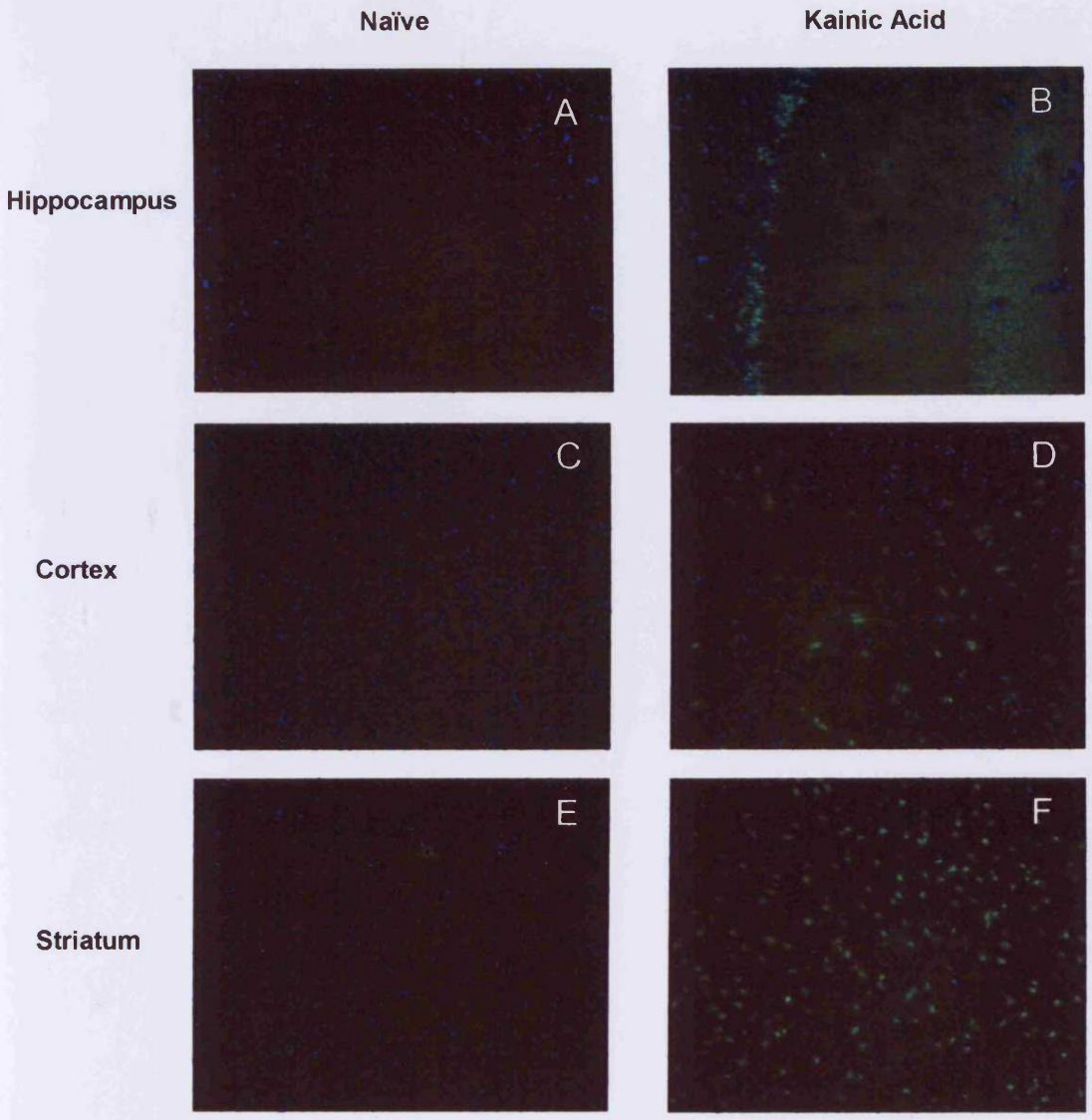


Figure 5.6: Kainic acid induces apoptosis in the hippocampus, cortex and striatum. Tissue cryosections were fixed in acetone and stained with caspACE. No staining was seen in naïve animals in any region of the brain (panels A, C and E). Strong staining was seen following kainic acid treatment in the hippocampus (B), cortex (D) and striatum (F). Magnification x10.

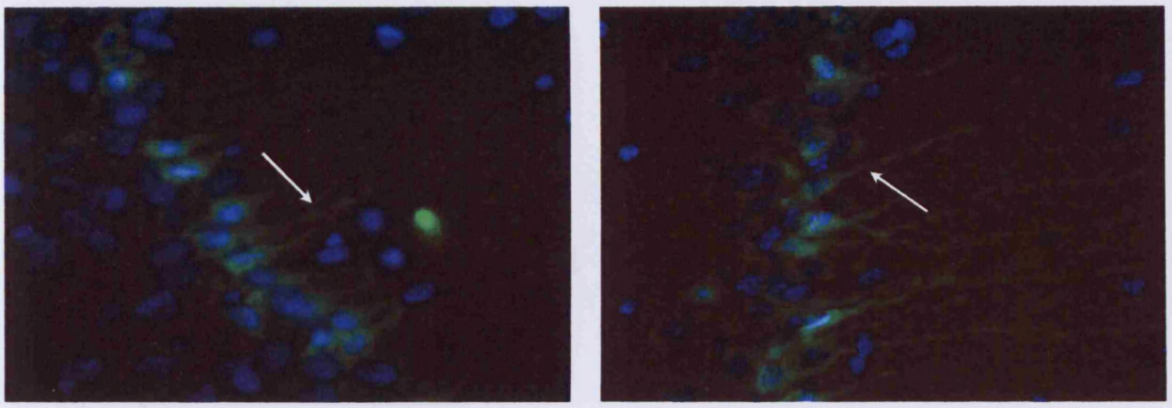


Figure 5.7: Kainic acid induces neuronal apoptosis. Tissue sections were stained with caspACE (examples from two animals shown). Apoptotic neurons were identified by the presence of caspACE staining and axonal extension from the CA1 layer (white arrows). Magnification x40.

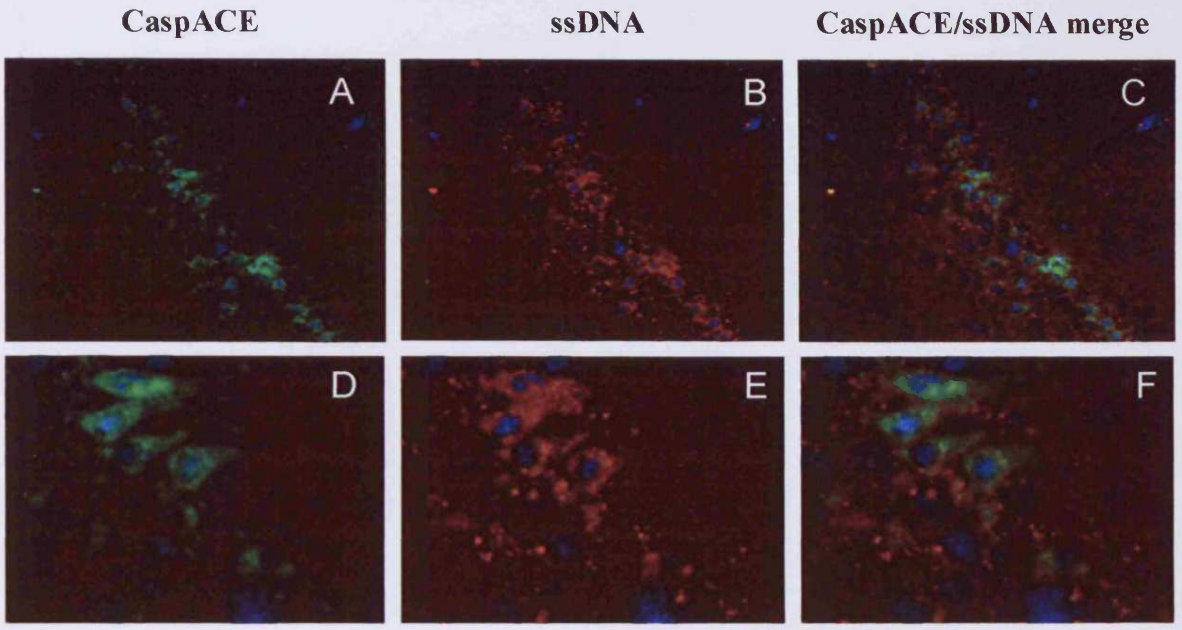


Figure 5.8: Kainic acid induces apoptosis in the hippocampus. Tissue sections from the CA1 region of the hippocampus were double stained with caspACE and anti-ssDNA mAb. Both apoptosis detection methods stained the same cells in each section (A, B x20; D, E x40). Merged images confirmed that these were indeed the same cells, demonstrating that they were apoptotic (C x20, F x40).

and scored for extent of tissue staining. Figure 5.9 shows representative hippocampus sections from each animal in both $C6^{-/-}$ and $C6^{-/-}$ + human C6 treated groups; paired littermates were used to compare reconstituted and control animals. Table 5.1 shows the clinical and apoptotic scores for all animals. In the $C6^{-/-}$ + human C6 group, 5/6 animals had some staining in the hippocampus, although in one this was low (KA 5); these animals also had staining in the cortex and striatum. In the $C6^{-/-}$ group, the majority of animals had less staining than their human C6 reconstituted littermate controls, with 3/6 animals having no detectable caspACE staining. One animal in the $C6^{-/-}$ group showed strong staining in all parts of the brain (KA 1), implying that MAC was not necessary to induce maximal damage. However, overall, more caspACE staining was seen in $C6^{-/-}$ + human C6 than in $C6^{-/-}$ alone, and suggested that MAC was promoting neuronal apoptosis in KA treated animals. Quantification using density slicing on Openlab software demonstrated that although higher apoptosis indices occurred in C6 reconstituted rats, there was no significant difference between groups (figure 5.10A). In addition, the amount of caspACE staining observed in each animal appeared to correlate with the maximum clinical score, as animals with a score of 2 or 3 had low levels of staining compared to those with a score of 4 (figure 5.10B).

3.7. Apoptotic neurons bind more C activation products *in vivo*

Tissue sections were next stained for C activation products using an antibody to C3c, which detects C3b and iC3b on cells. Figure 5.11 shows that C3c staining co-localised with caspACE staining, demonstrating that apoptotic neuronal cells in the hippocampus bound more C activation products. Staining with the secondary antibody only was negative (figure 5.11E).

3.8. Neuronal apoptosis is associated with altered expression of rat CReg *in vitro*, and *in vivo*

Rats possess the CRegs CD55 and CD59, in common with humans, but in place of CD46, which is restricted to the testis in rats (Mizuno, Harris et al. 2004), have a molecule known as Crry which has

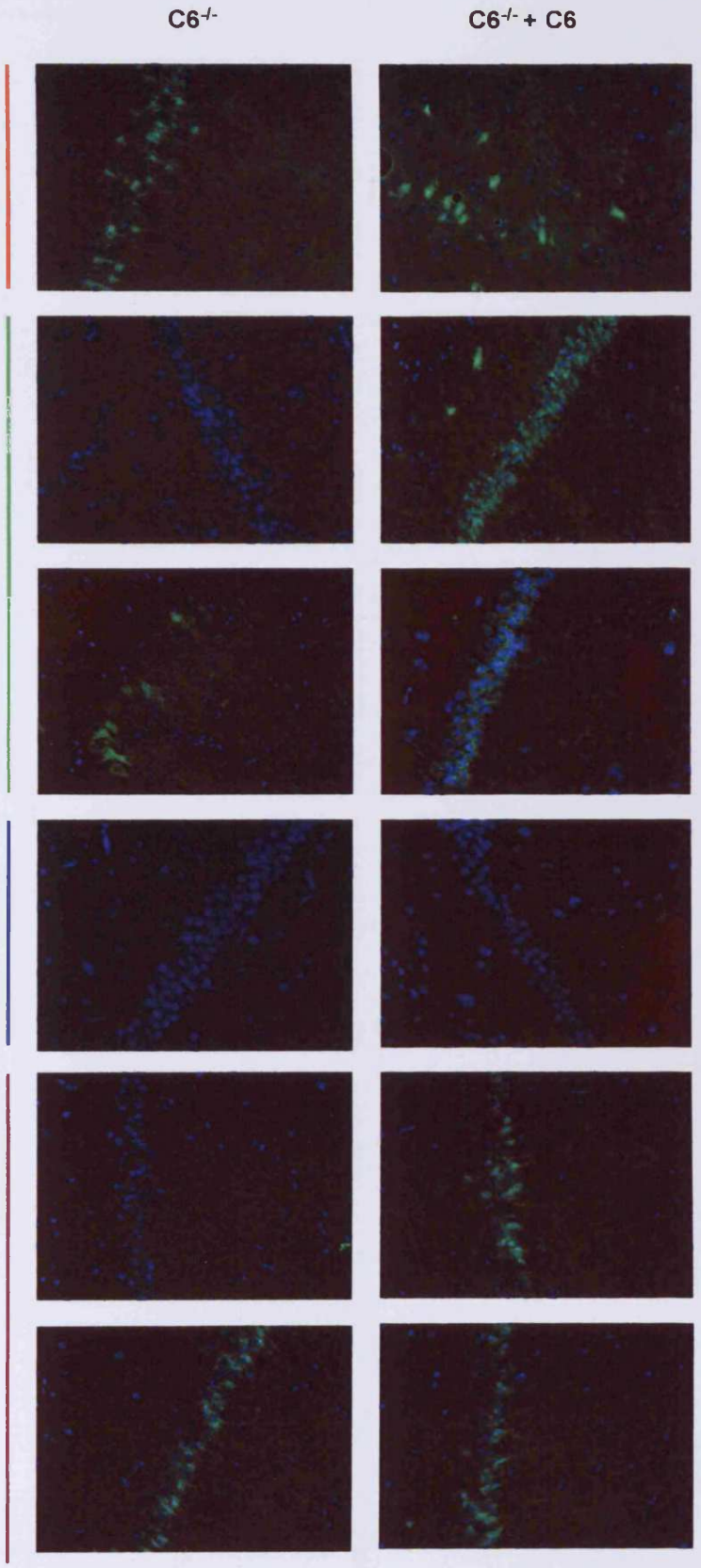


Figure 5.9: Fewer $C6^{-/-}$ animals showed neuronal apoptosis than $C6^{-/-}$ + C6 following KA treatment. Hippocampus caspACE staining was examined in all animals. Staining was seen in 3 of the 6 $C6^{-/-}$ animals, and 5 of the 6 $C6^{-/-}$ + C6 animals. Coloured bars indicate matched littermate pairs. Representative sections from individual animals. Magnification x20.

Animal	Add-back	Max Clincal score	Hippocampus caspACE staining	Cortex caspACE staining	Striatum caspACE staining
Naïve	Nil	0	-	-	-
KA 1	PBS	4	+++	++	++
KA 2	C6	4	+++	+	++
KA 3	PBS	4	++	+	++
KA 4	PBS	2	-	-	-
KA 5	C6	4	+	+	+
KA 6	C6	4.5	+++	++	++
KA 7	C6	3	-	-	-
KA8	PBS	2	-	-	-
KA9	C6	4	++	++	++
KA 10	C6	4	++	++	++
KA 11	PBS	3	-	-	-
KA 12	PBS	4	++	++	++

Table 5.1: MAC promotes KA-induced neuronal apoptosis. Staining was examined in the hippocampus, cortex and striatum of all animals. The table shows only animals with a clinical score of 4 or more showed clear staining with caspACE. Staining was strongest in the hippocampus. – no cells stained, + few cells stained, ++ cells stained, +++ large number of cells stained.

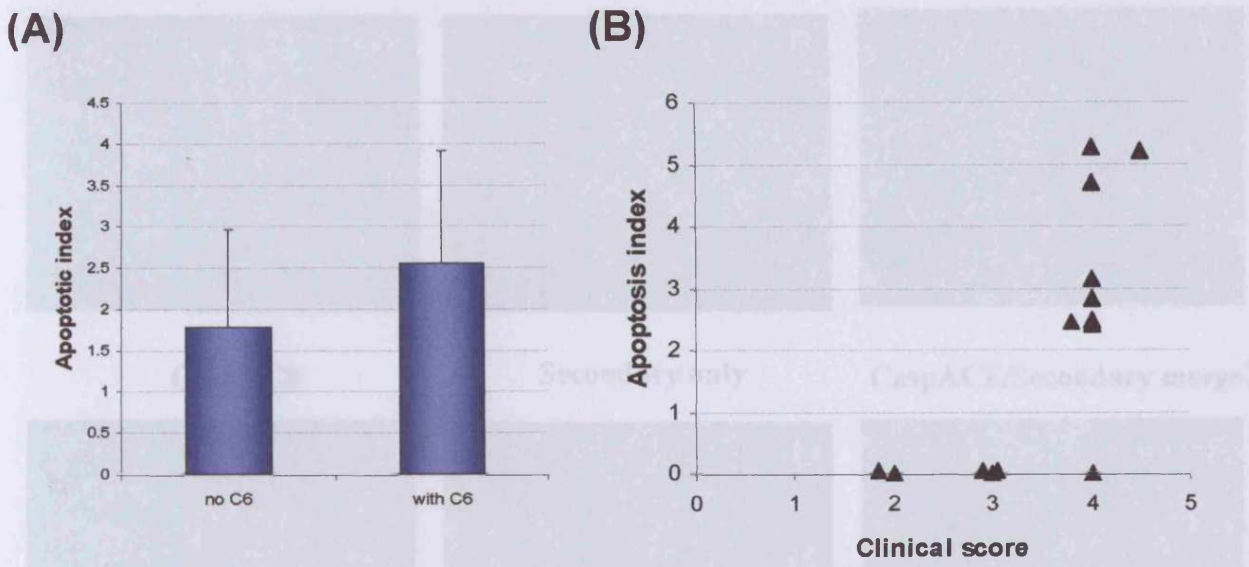


Figure 5.10: Quantification of caspACE staining on tissue sections. **(A)** Sections were stained with caspACE and the area stained was evaluated using density slicing and calculating the total stained area. More staining was seen in animals reconstituted with C6, but the difference was not significant as assessed using the t-test. n=6 for each group, and data are expressed as +/- SEM. **(B)** Apoptosis index was plotted against clinical score for all animals. Only animals with a clinical score of 4 or more stained for apoptotic cells. One animal with a score of 4 did not stain for apoptotic cells.

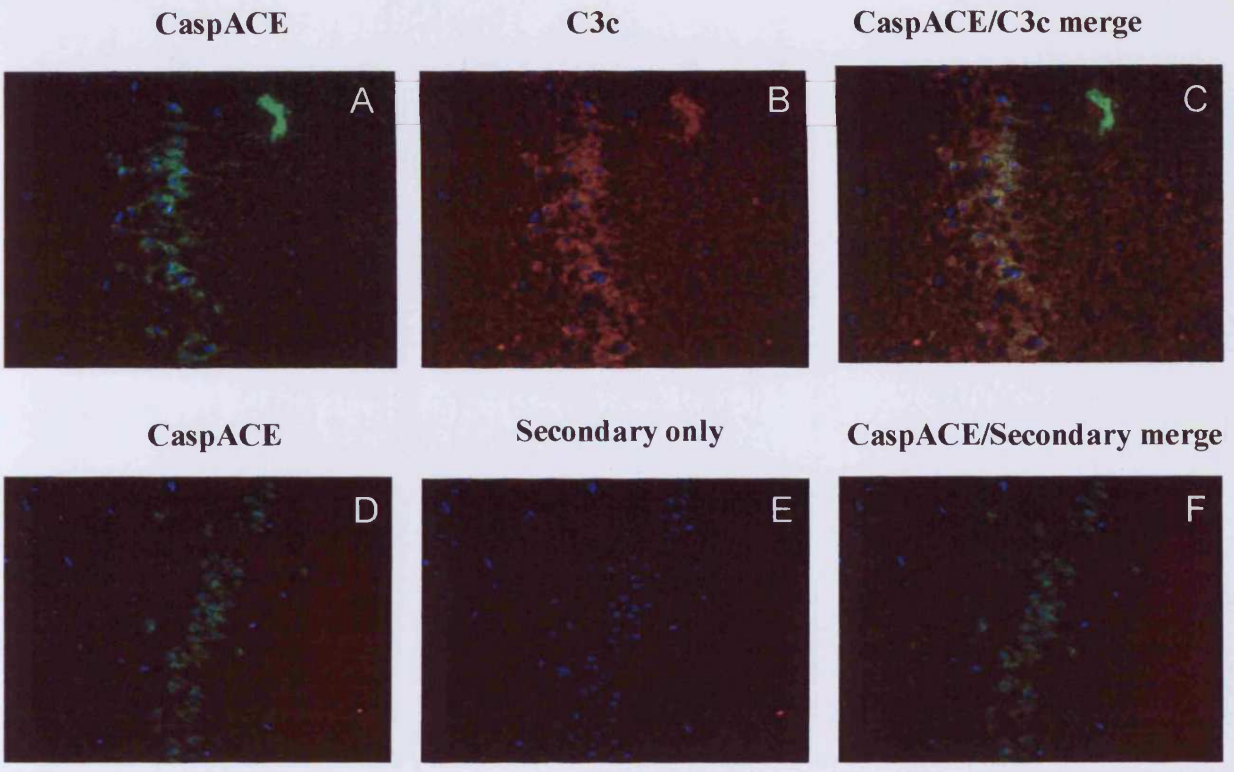


Figure 5.11: Apoptotic neurons activate C in KA treated animals. Cryosections from KA treated animals were double stained with caspACE **(A, D)** and either anti-C3c **(B)** or secondary Ab only **(E).** C3c staining, but not secondary Ab only staining, occurred clearly on caspACE positive cells, and was confirmed on merged images **(C)** compared with secondary Ab **(F).**

both cofactor and decay-accelerating activities. In order to assess whether rat neuronal cells also lose CReg during apoptosis, the rat neuroblastoma cell line B50 was used, and apoptosis induced using UV irradiation. Figure 5.12A shows that UV induced apoptosis in this cell line, as assessed by annexin-V-FITC and PI staining, and figure 5.12B shows that all three CReg were lost during apoptosis. The pattern of loss varied between the different CReg, with diffuse loss of CD59, but clearer reduction in both CD55 and Crry. Crry expression was reduced by 55%, and CD55 by 90%. CD59 expression was reduced by 40% over the whole population.

Sections were next stained for CRegs Crry, CD55 and CD59, in order to establish whether the phenomenon of CReg loss from apoptotic neurons observed in vitro also occurred in vivo. Figure 5.13 shows caspACE double staining for CD93 (endothelial cells and microglia), tomato lectin (microglia), and glial fibrillary acidic protein (GFAP; astrocytes) and CRegs. Crry staining was diffuse and CD55 staining was weak; neither CReg followed the staining patterns of the cell markers. CD59 staining, in contrast, was strong and showed high levels of staining on cells that had a similar pattern to CD93 and tomato lectin, indicating that these cells were likely endothelial cells and microglia. A more diffuse pattern was also observed that was suggestive of astrocytic staining, but may have been related to oligodendrocytes or axonal bundles.

Figure 5.14 shows caspACE and CD59 staining of sections from control animals and KA treated animals; CD59 gave stronger staining in KA treated animals. On hippocampal neurons, diffuse staining was observed in the naïve control, indicating that these cells possessed CD59. In the KA treated animals, both with and without C6 reconstitution, caspACE positive cells were surrounded by strong staining for CD59, but no co-localisation was observed. This suggested that cell surface staining for CD59 may be increased, or that these cells had shed CD59 into their immediate

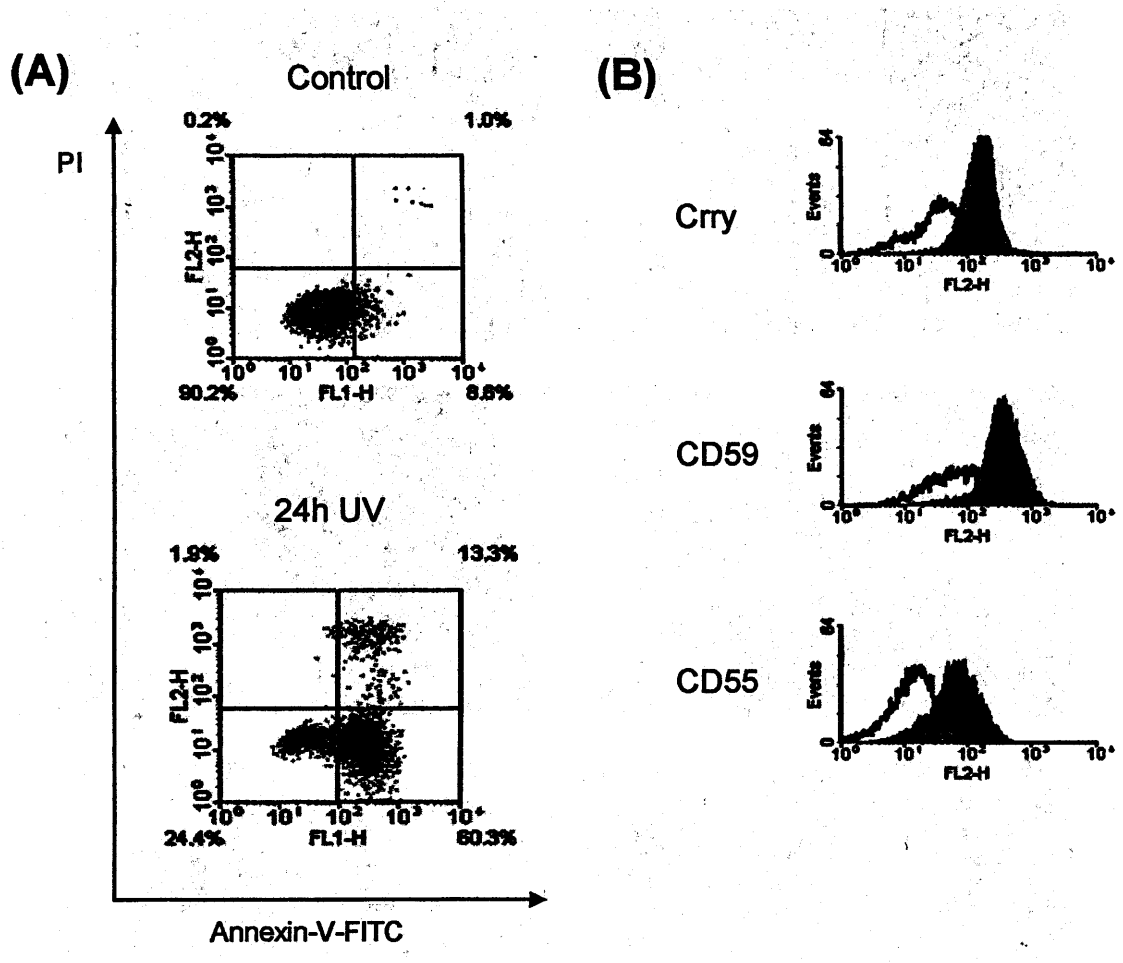


Figure 5.12: Apoptotic rat B50 neuroblastoma cells lose cell surface CReg. (A) B50 rat neuroblastoma cells were exposed to UV (400mJ) and incubated for 24h. Cells were stained with annexin-V-FITC/PI. After 24h, 60% of cells were in early (annexin+/PI-) apoptosis and 13% were in late (annexin+/PI+) apoptosis. **(B)** CReg loss occurred from UV treated cells. Grey filled profile represents the control population; the black line profile represents the UV treated population. Cell surface expression of all CRegs was reduced following UV treatment.

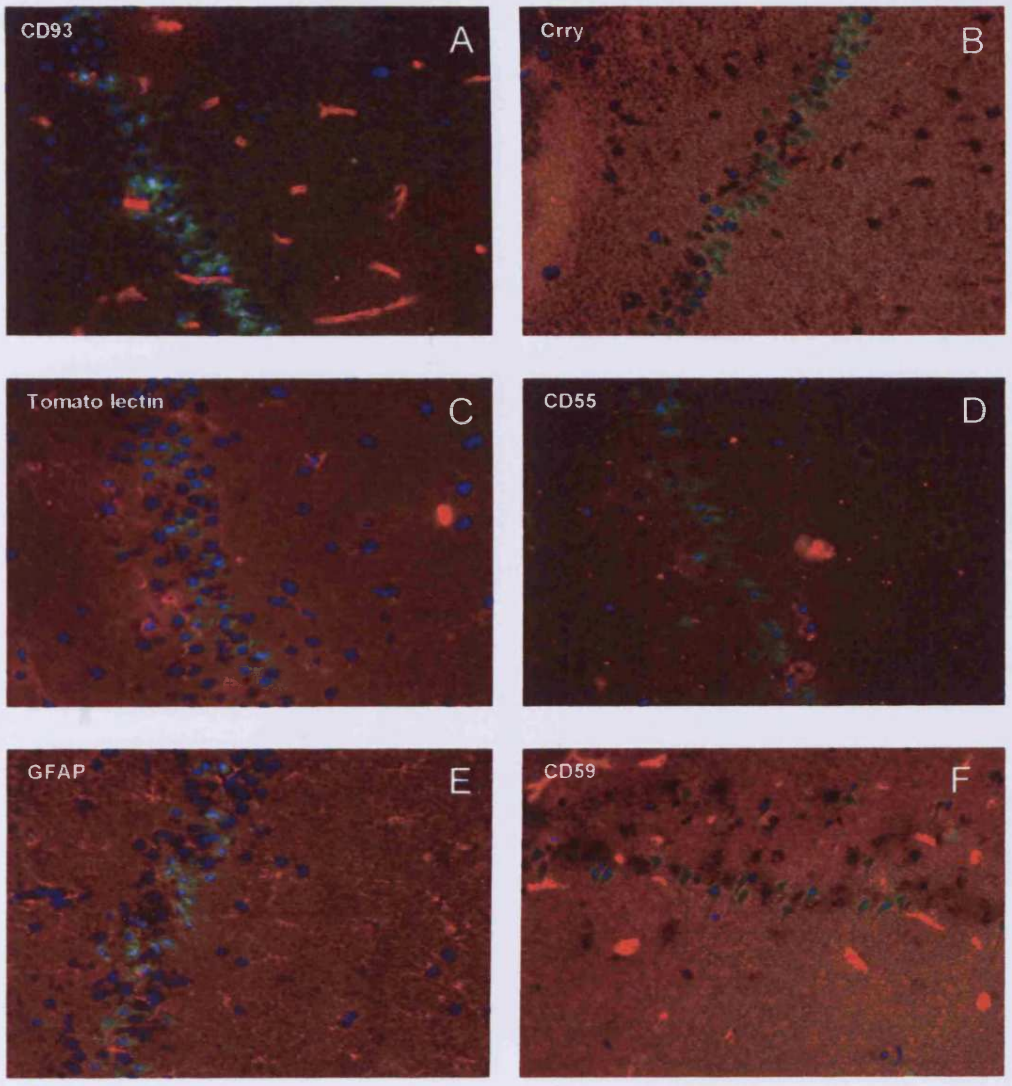


Figure 5.13: Cell markers and CReg staining in the hippocampus. Sections were stained with antibodies against cell markers (CD93 for endothelial cells and microglia; tomato lectin for microglia and macrophages, and GFAP for astrocytes) or CRegs, and caspACE for apoptotic cells. None of the cell markers co-localised with caspACE stained cells (**A**, **C**, **E**). Crry staining was diffuse, and did not follow the staining patterns of the cell markers (**B**). CD55 staining was very low, and did not follow the patterns seen with the cell markers (**D**). CD59 staining was strong, and stained cells in a similar pattern to CD93, tomato lectin and also GFAP. Blue: DAPI, green: caspACE, red CReg or cell marker.

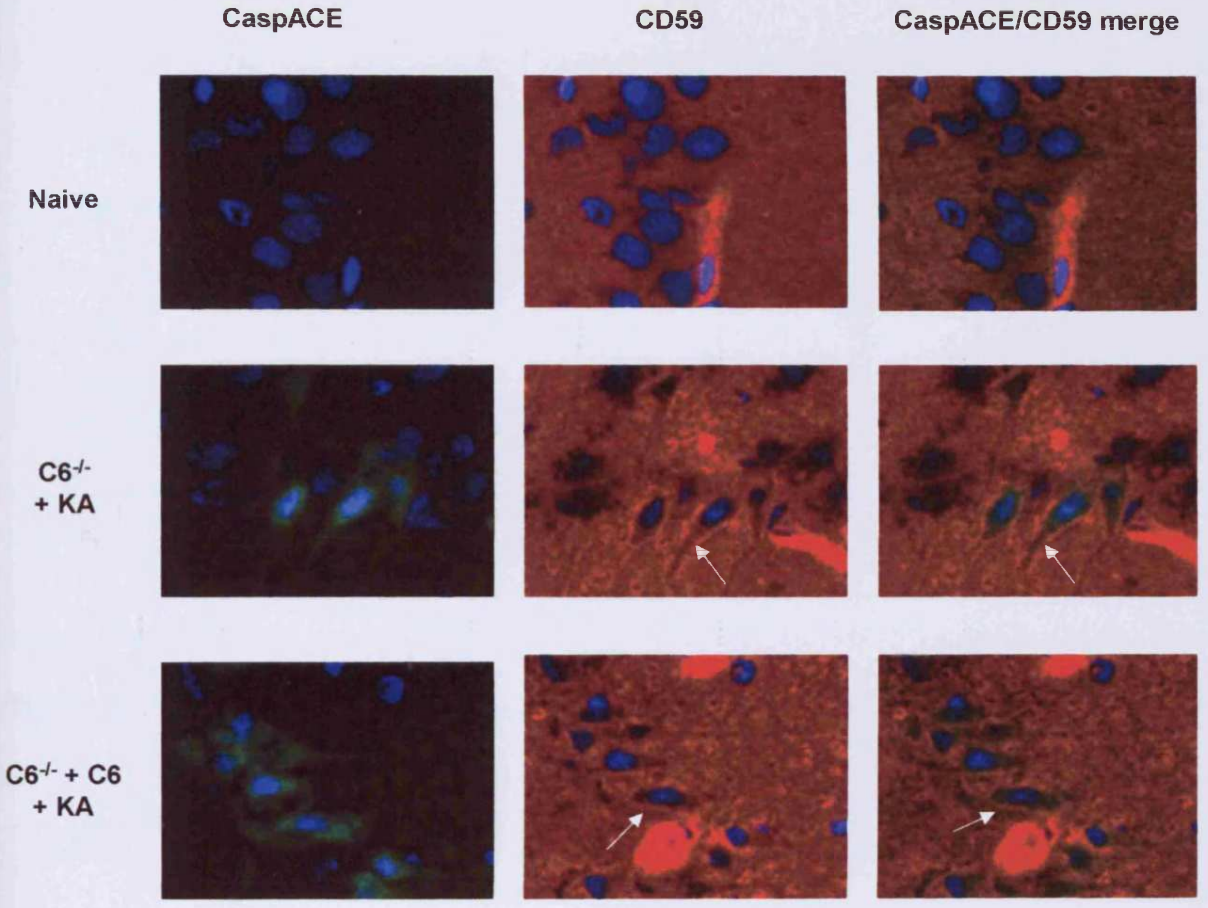


Figure 5.14: CD59 expression on apoptotic neurons. Cryosections from naïve and KA treated animals were stained with caspACE (green), to identify apoptotic cells, anti-CD59 mAb (red), and DAPI (blue). Naïve animals showed no apoptotic cell staining, and low levels of CD59 staining. KA treated animals showed clear populations of apoptotic cells, and higher CD59 staining. Cells with strong CD59 staining were clearly visible, likely microglia (rounded cells) or endothelial cells (elongated cells). Apoptotic cells showed high levels of staining around the cells, possibly indicating high cell surface expression or shedding into the environment immediately surrounding the cells. Merged images showed that this staining did not co-localise with caspACE staining. White arrows indicate CD59 staining outlining caspACE positive cells.

environment. In addition, areas were noted where there was no caspACE staining or CD59 staining. These areas had irregular nuclei, possibly representing cells that had died and were necrotic. In KA treated animals, increased numbers of more strongly positive cells were observed, possibly indicating increased microglial activation.

Crry staining was diffuse in naïve animals, and in KA treated animals was similar, but with areas of reduced staining (figure 5.15). Merged caspACE/Crry images showed caspACE positive cells occupied the areas of reduced Crry staining, while co-localisation of caspACE and Crry occurred at the periphery of the cells, possibly indicating localisation at the cell surface of apoptotic cells. CD55 staining was much weaker than Crry or CD59 staining (figure 5.16), and showed areas of slightly increased staining KA treated animals compared to naïve animals. Merged images showed that this occurred on caspACE positive cells, indicating that these cells may have up-regulated CD55 expression.

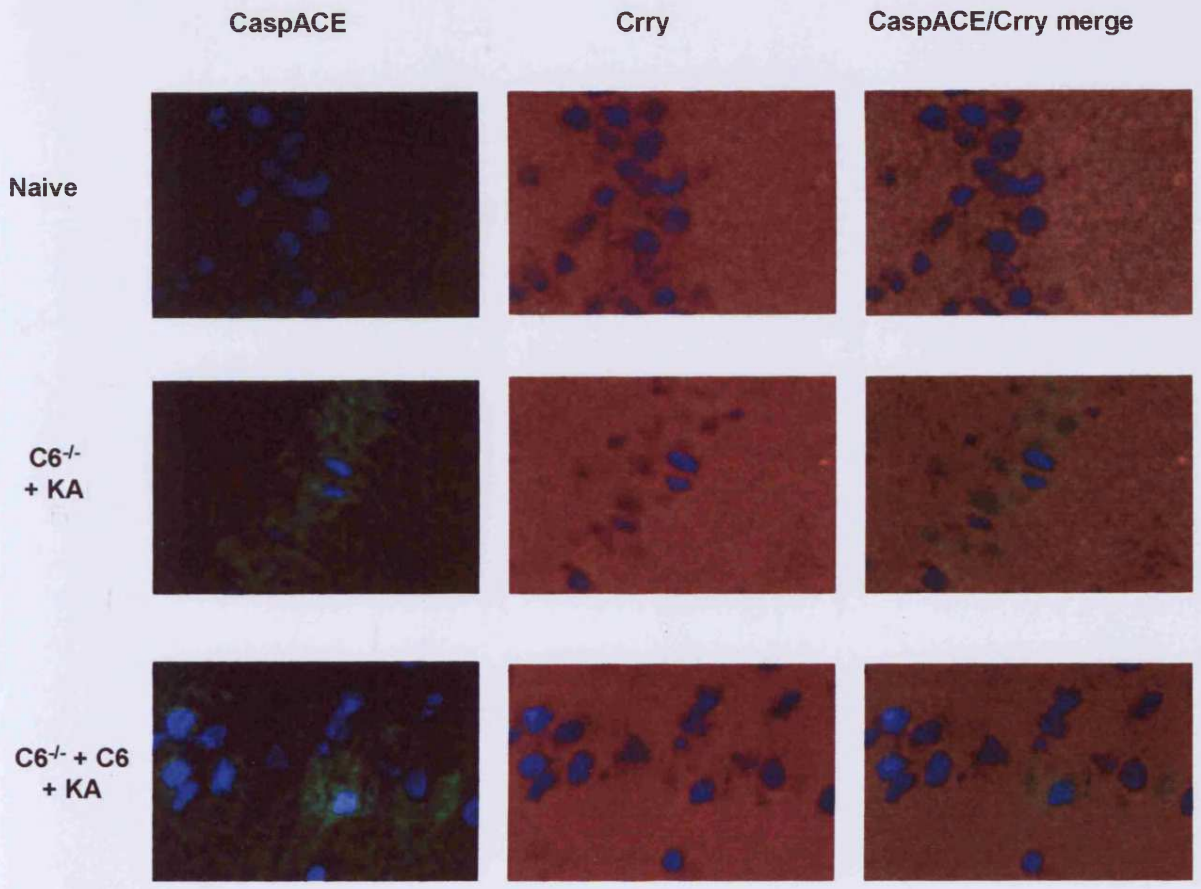


Figure 5.15: Crry expression on apoptotic neurons. Cryosections from naïve and KA treated animals were stained with caspACE (green) to identify apoptotic cells, anti-Crry mAb (red) and DAPI (blue). Naïve animals showed no apoptotic cell staining, and low levels of diffuse Crry staining. KA treated animals showed clear populations of apoptotic cells, and similar Crry staining to naïve animals, but areas of low staining were observed in both C6 deficient rats and reconstituted animals. Merged images showed that Crry staining co-localised with caspACE staining on the periphery of cells (yellow in merged images).

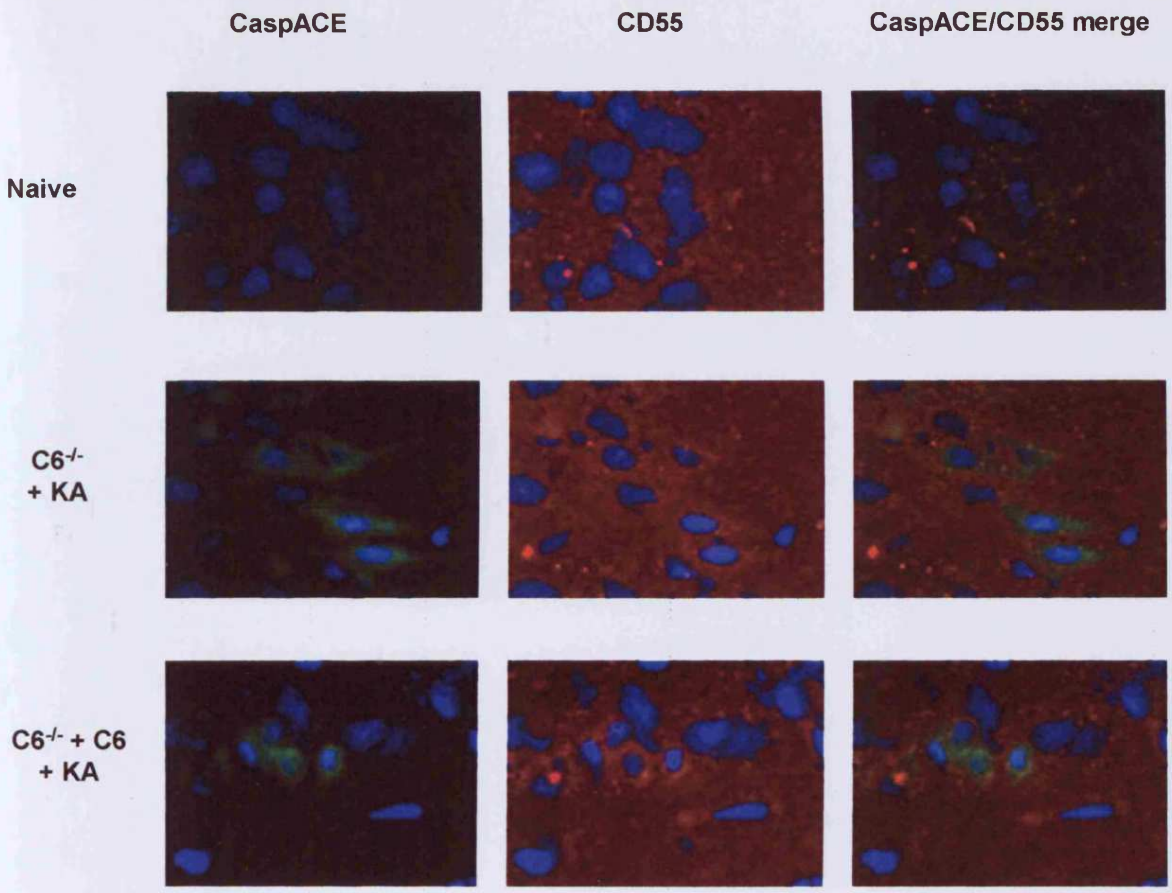


Figure 5.16: CD55 expression on apoptotic neurons. Cryosections from naïve and KA treated animals were stained with caspACE (green), to identify apoptotic cells, anti-CD55 mAb (red) and DAPI (blue). Naïve animals showed no apoptotic cell staining, and low levels of CD55 staining. KA treated animals showed clear populations of apoptotic cells, and slightly increased CD55 staining in and around some cells. Merged images showed that some CD55 staining co-localised with caspACE staining (yellow in merged images).

4. Discussion

Excitotoxic neuronal death occurs in a wide range of neurological diseases, including MS, AD, stroke and epilepsy. Under these conditions, both apoptosis and necrosis occur, and death is induced by both intrinsic factors, such as large changes in Ca^{2+} flux via glutamate channels, and extrinsic factors, such as activation of microglia and astrocytes. Excitotoxic models of neuronal cell death have been shown to involve C activation and neuronal apoptosis (Pasinetti, Tocco et al. 1996; Gilliams-Francis, Quaye et al. 2003), and thus is a useful approach to studying the interaction of these two processes in vivo, in order to understand more fully how they interact in human disease.

The rat KA model was chosen for the study since it has been well characterised both in terms of the clinical features the animals display, and the neuropathological consequences. Various studies have shown that neuronal apoptosis in the hippocampus is an important feature of this model (Puig and Ferrer 2002; Gilliams-Francis, Quaye et al. 2003), and this was confirmed in the present study using a combination of a fluorescent caspase inhibitor (FITC-zVAD-fmk) and antibodies against ssDNA, that co-localised on double staining. The hippocampus is the most sensitive area to KA, particularly the CA1 and CA3 regions (Sperk, Lassmann et al. 1985). With larger doses, the cortex and striatum become involved. My study confirmed this, with caspACE positive cells present in the hippocampus, and scattered apoptotic cells particularly in the striatum and cortex. These changes were only seen in animals that had developed seizures following KA administration, in agreement with other studies. Indeed, seizures appear to be crucial in the pathophysiology of neuronal damage, since both seizures and neuronal loss can be blocked by the administration of diazepam (Pollard, Charriaut-Marlangue et al. 1994).

KA-induced damage in the brain is also associated with alterations in the presence and activation status of a variety of immune cells, including both those intrinsic to the brain and blood-borne (Campbell, Finlay et al. 2004). Changes in cellular infiltration appear 24 hours after KA administration and peak after 3-5 days. Typically, an astrogliosis is seen, with increased numbers of microglial cells, and small numbers of macrophages and lymphocytes that enter via the disrupted blood-brain barrier. In this study, 24 hours was chosen as the end point as the aim was to study the early events of neuronal apoptosis and C activation without the complicating factors introduced by infiltrating cells.

A key hypothesis in this study is that the MAC is neuroprotective under excitotoxic conditions in vivo. To this end, $C6^{-/-}$ rats were used in the model, with or without reconstitution with human C6. The human C6 used was not totally pure, despite attempts to remove contaminants using gel filtration chromatography. This was not a significant problem, since the KA model is acute, and the contaminants were unlikely to be toxic or cause problems in generating immune responses over this time frame. In addition, the human C6 itself was active, and reconstituted haemolytic activity in $C6^{-/-}$ rat serum both in vitro and in vivo. Using the add-back method, the haemolytic activity of $C6^{-/-}$ rat serum was reconstituted in vivo to a CH50 of 50% maximum at 24h, sufficient to form MAC following C activation.

The effect of the MAC on KA treated rats was examined using clinical scores and assessment of the number of apoptotic cells in the hippocampus in the two groups. The clinical scores did not show a significant difference between those animals that had been reconstituted with C6 and those that hadn't. When the extent of apoptosis was examined, the reconstituted animals appeared to have more apoptosis than their $C6^{-/-}$ counterparts, suggesting that the MAC was causing more damage in these animals, but quantification using imaging analysis software and statistical evaluation showed no

significant difference. However, in $C6^{-/-}$ rats that had developed seizures and hippocampal damage, a similar degree of neuronal apoptosis was noted as in the $C6^{-/-}$ that had been reconstituted with C6. Thus the MAC may sensitise neurons to excitotoxic damage, but once a threshold is exceeded and seizures occur, neurons begin to die by apoptosis regardless of whether MAC is present or not. This hypothesis now needs further investigation. Such an effect would be the converse of studies that have shown fleeting stimulation of neuronal glutamate receptors sensitises the cells to MAC-mediated cell death in vitro (Xiong and McNamara 2002). It is possible that both MAC-mediated and excitoxin-induced death may each sensitise to the other as both may kill cells as a result of calcium overload. Indeed, MAC components, when infused directly into the mouse brain, induce seizures and neuronal damage when the MAC is fully formed (Xiong, Qian et al. 2003). It is possible that MAC-mediated Ca^{2+} influx acts synergistically with Ca^{2+} influx through glutamate activated channels to promote seizure activity, and thus neuronal apoptosis, in the KA model. Importantly, it appears that the neuroprotective effect of C5, noted in C5-deficient mice, is most likely entirely due to C5a, which probably acts to counter-act MAC-mediated sensitisation to seizures. Thus the hypothesis set out in the introduction is not true: MAC is not neuroprotective under these conditions in vivo, and is more likely damaging in the acute phase. This finding has important implications for the use of C therapeutics, since agents that specifically target the MAC, and leave C5a intact, may reduce the degree of excitotoxic damage, and allow C5a to perform its role as an anti-apoptotic neuroprotectant. However, this study needs to be repeated to confirm the trends observed, since no statistically significant difference was found.

I have previously shown that apoptotic neuronal cells lose CRegs and consequently activate C more readily, and that this leads to increased phagocytosis (Chapter 3). The next stage of this investigation was to establish whether this also occurred in rat neuronal cells in vitro, and in vivo following KA challenge. Apoptosis in rat B50 neuroblastoma cells resulted in the loss of Crry, DAF and CD59,

similar to the human IMR-32 cell line, confirming that this effect was also a feature of neuronal apoptosis in the rat. Although C activation on apoptotic B50 cells was not examined, there was clear co-localisation of C activation products with apoptotic neuronal cells in all areas of the brain in vivo, indicating that these cells did indeed become opsonised more readily. In such an acute situation, it is likely that C plays an important role in clearing apoptotic debris, not only by opsonising dying cells, but also in attracting phagocytic cells to the damaged area. However, clear evidence of phagocytosis was not apparent at the time point examined here, but would be an important line of work in the future.

CReg loss from neuronal cells was clearly shown in vitro. In vivo, CD59 expression was increased in KA treated animals, with an increase in the number of cells expressing high levels of CD59, suggesting that microglia were beginning to accumulate in the damaged areas. In the hippocampus, apoptotic neuronal cells appeared to be surrounded by high levels of CD59 staining, but this did not co-localise with the apoptotic cell itself. This suggested that CD59 may have accumulated in the environment immediately around the apoptotic cell, a finding in keeping with CD59 shedding, but these findings are also consistent with increased expression of CD59 on the neuronal cell surface. It was also possible that the apoptotic cell was becoming engulfed by cells with processes bearing high CD59 levels, but this was less likely, since all apoptotic cells were surrounded by high CD59 staining, and no phagocyte cell bodies were evident by lectin staining. However, co-localisation studies with CD59 and cell-specific markers would be needed to confirm this.

Crry staining was diffuse in both untreated and KA treated rats, with areas of low level staining that corresponded to apoptotic cells in the latter. This finding was in keeping with the loss of Crry during apoptosis of rat neuroblastoma cells. Interestingly, Crry and caspACE co-localised at the periphery of apoptotic cells, suggesting that Crry may be localised at the membrane of apoptotic cells, although

the staining was not clear and needs repeating. CD55, in contrast, was expressed only at a very low level, and slightly increased staining was observed on apoptotic cells, possibly reflecting increased CD55 expression on these cells. However, other studies have shown that CD55 is upregulated on neurons following chronic, but not acute inflammatory insults (van Beek, van Meurs et al. 2005). The Crry and CD55 findings must be interpreted with caution, as the quality of staining was not optimal and need repeating.

The data presented suggest that C activation and neuronal apoptosis are important during excitotoxic insults in the brain. The trends noted suggest that the MAC may sensitise neurons to the effects of excitotoxins acting on glutamate receptors, implying that when MAC is present in Alzheimer's plaques or MS lesions it may be damaging even at sublytic doses. This hypothesis requires further investigation. C activation occurs on apoptotic neurons, possibly as a result of loss of CReg, thus targeting them for clearance, but may also be a focus for the damaging effects of C, as we have seen. However, the data, taken as a whole, suggest that therapeutics targeted specifically at the MAC may be beneficial in reducing the effects of excitotoxicity in many neurological diseases, leaving the beneficial effects of C opsonisation in aiding apoptotic cell clearance, and the anti-apoptotic effects of C5a, intact, while removing the deleterious effects of the MAC-mediated damage.

Chapter 6: Discussion and Conclusion

1. Summary of main findings

This thesis has been concerned with the interaction of neuronal apoptosis and C activation in CNS disease. Both occur in various neurological disorders at sites of active pathology (Compston, Morgan et al. 1989; Lue, Brachova et al. 1996; Prineas, Kwon et al. 2001), both are important in determining whether neurons live or die (Singhrao, Neal et al. 2000; Meyer, Weissert et al. 2001), and in deciding whether death exacerbates inflammation. In the Introduction, I laid out the hypothesis for the study:

The C system modulates neuronal cell turnover in the CNS

This was investigated in a number of ways, involving in vitro and in vivo approaches. Two primary modes of cell death were examined: apoptosis and necrosis. The former is classically anti-inflammatory, and the latter pro-inflammatory; the first part of the study therefore examined the role of C in modulating the fate of neuronal cells that had already initiated the apoptotic programme, either promoting phagocytosis or modulating susceptibility to MAC-mediated lysis. The focus of the work was in identifying the factors that were responsible for altering C activation on apoptotic neuronal cells; the findings are summarised in figure 6.1. This work revealed that the CRegs CD46 and CD59 were shed during apoptosis, CD46 by an MMP-mediated cleavage event, and CD59 on apoptotic blebs and as a soluble form. The mechanism of generation of soluble CD59 from apoptotic cells was not followed up, and requires further investigation. C activation was increased on apoptotic cells, and this was reduced by blocking the MMP-mediated loss of CD46. As a result, apoptotic

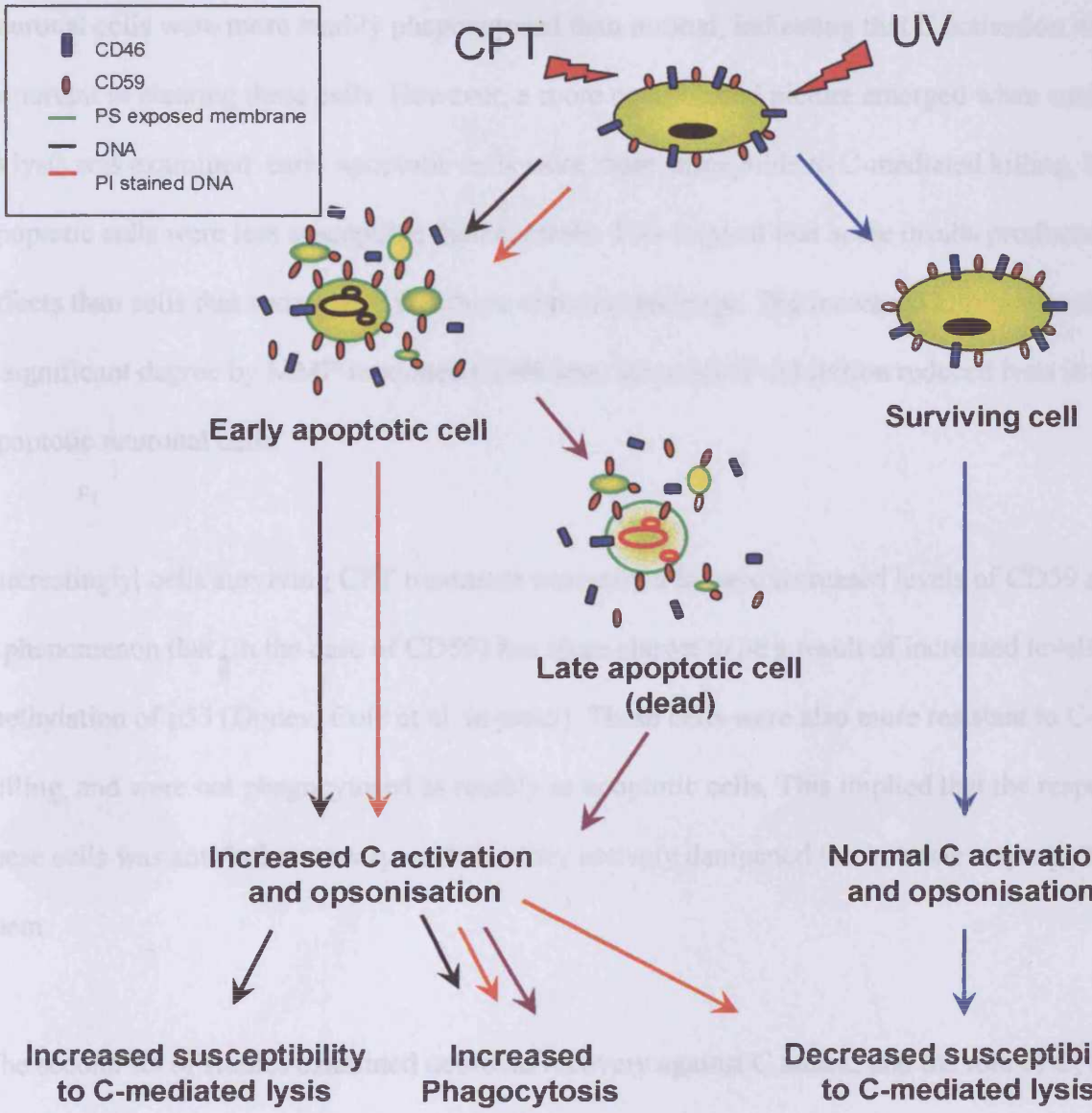


Figure 6.1: Proposed interaction between C and neuronal cells exposed to pro-apoptotic stimuli. Apoptotic stimuli (CPT and UV irradiation) result in a population of surviving cells and early apoptotic cells. A variety outcomes are possible, the balance of which will likely depend on the severity of the apoptotic insult, and the proximity of phagocytic cells. Blue arrows indicate the consequences for surviving cells; black arrows the consequences for cells undergoing apoptosis early after the insult; orange arrows indicate the fate for cells undergoing delayed apoptosis after the insult; and purple indicates the consequences for cells in late apoptosis/secondary necrosis. CD59 levels increase on surviving cells; CD46 levels are unchanged. Early apoptotic cells lose CD46 (soluble) and shed CD59 (blebs and soluble), and begin to lose CD59 from their cell surface, with 30% drop by the time they are in late apoptosis.

neuronal cells were more readily phagocytosed than normal, indicating that C activation was important in clearing these cells. However, a more complicated picture emerged when susceptibility to lysis was examined: early apoptotic cells were more susceptible to C-mediated killing, but later apoptotic cells were less susceptible than controls. This implied that acute insults produced different effects than cells that succumbed to a more chronic challenge. The increased killing was mediated to a significant degree by MMP-mediated CD46 loss, since MMP-inhibition reduced lysis in early apoptotic neuronal cells.

Interestingly, cells surviving CPT treatment were noted to have increased levels of CD59 and CD200, a phenomenon that (in the case of CD59) has since shown to be a result of increased levels and methylation of p53 (Donev, Cole et al. in press). These cells were also more resistant to C-mediated killing, and were not phagocytosed as readily as apoptotic cells. This implied that the response of these cells was anti-inflammatory, and that they actively dampened the immune response towards them.

The second set of studies examined neuronal recovery against C attack, and the role of C, and in particular the MAC, in promoting or inhibiting apoptotic and C-mediated neuronal cell death (summarised in figure 6.2). Sublytic doses of MAC have been shown to induce cellular protection against lytic doses of MAC and other pore formers (Reiter, Ciobotariu et al. 1995), and also promote or inhibit apoptosis in various cell types (Soane, Cho et al. 2001; Nauta, Daha et al. 2002), findings that may also extend to neuronal cells. First, a reactive lysis system using purified MAC components was developed, in order to isolate MAC effects from confounding factors in serum. This demonstrated that the cell line used for the in vitro work, the IMR-32 human neuroblastoma line, was resistant to MAC-mediated death when MAC was formed using reactive lysis. Real-time calcium flux assays using reactive lysis demonstrated that these cells were being effectively attacked, as calcium

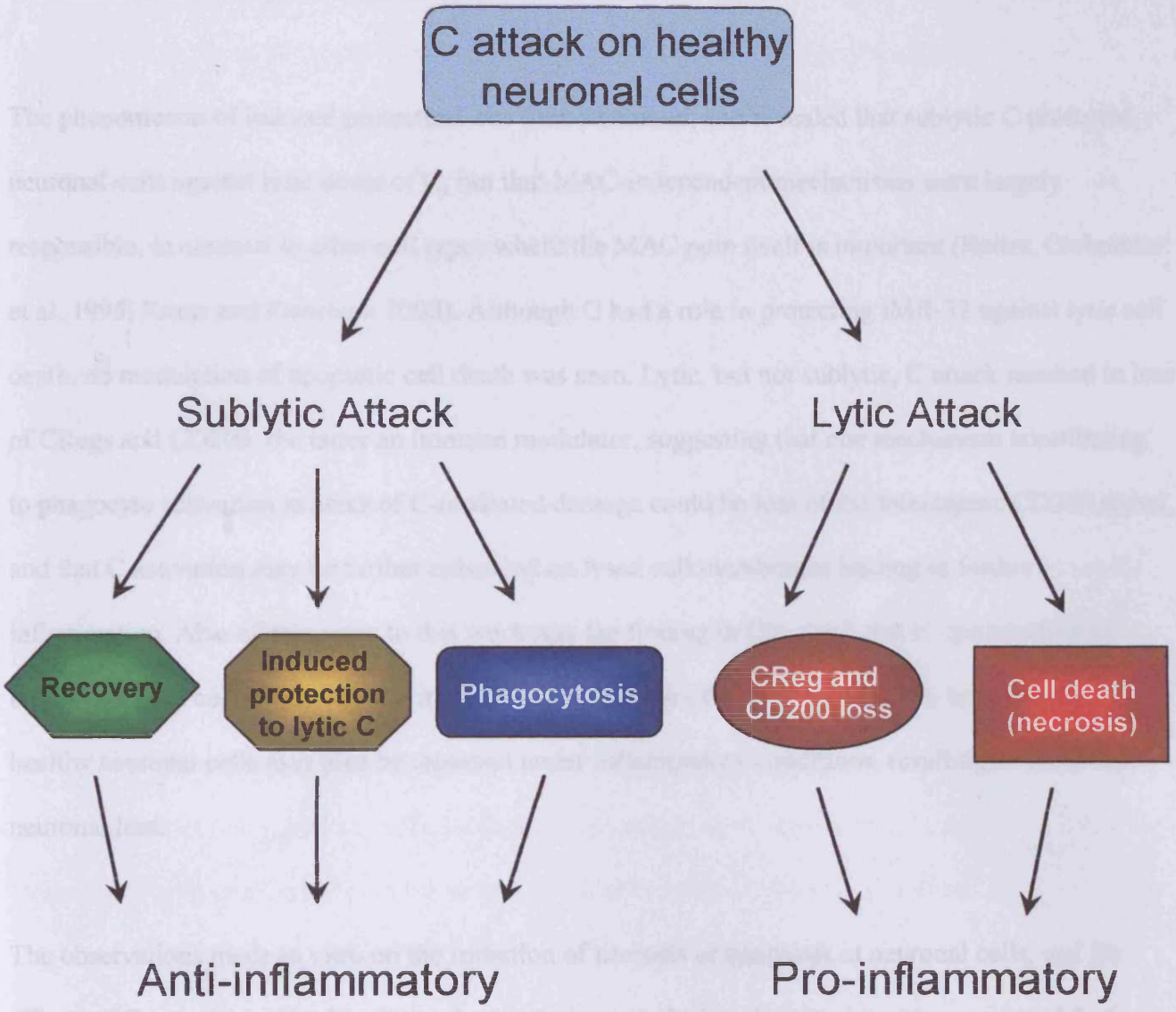


Figure 6.2: Summary of effect of C attack on healthy neuronal cells. C attack has a number of effects on neuronal cells as investigated in this thesis. Lytic attack results in necrotic cell death and loss of cell surface CD46, CD59 and CD200, all potentially pro-inflammatory. Sublytic attack results in activation of recovery mechanisms, MAC-independent induction of protection against subsequent lytic attack, and phagocytosis. The latter also likely occurs during lytic attack. Sublytic attack resulted in phenomena likely to preserve cellular integrity and removal of cells before death, and can therefore be seen as anti-inflammatory.

similar whether MAC was present or not.

Apoptotic neurons did activate C more readily than other neurons in vivo, in keeping with the in vitro data. Indeed, in the model used, C activation products were well localised to apoptotic neurons, indicating that opsonisation was quite specific. Preliminary studies on CReg expression showed high CD59 staining in the area immediately surrounding, but not co-localising with, apoptotic staining, suggesting that these cells either up-regulated CD59 or were shedding it into their environment. Crry staining was weak, and showed a reduction on apoptotic neurons, while CD55 showed a marginal increase on these cells.

Overall, these data give a broad-based picture of the interaction of neuronal apoptosis and C-activation. Neuronal cells are able to spontaneously activate C, but possess mechanisms to control C activation and resist C-mediated lysis. The MAC, in the acute setting, is damaging, not only in actively lysing cells, but in possibly promoting seizures and apoptosis following excitotoxic stimuli. In addition, in the early stages of apoptosis, C activation increases as neurons lose CRegs, and is increasingly damaging, as these cells become more susceptible to lysis. Under such acute situations, clearance of apoptotic debris is likely to be critical in limiting further inflammation.

The situation may be different if the insult is more chronic, or if the disease is in a recovery or repair phase, since under these circumstances sublytic C effects are likely to be relevant. In neurons, sublytic C induces protection to lytic C in a MAC-independent fashion. Neurons that survive an apoptotic insult become more resistant to C by up-regulation of CD59, and induce anti-inflammatory activity by up-regulating the immune modulator CD200. Thus cells that survive an initial onslaught, either C activation or an apoptotic insult, are more resistant to both apoptosis and C, and promote a response that facilitates recovery and repair.

flux increased when cells were exposed to the MAC, and that they possessed recovery mechanisms, since the calcium flux peaked and then returned to a baseline level.

The phenomenon of induced protection was then examined, and revealed that sublytic C protected neuronal cells against lytic doses of C, but that MAC-independent mechanisms were largely responsible, in contrast to other cell types where the MAC pore itself is important (Reiter, Ciobotariu et al. 1995; Kraus and Fishelson 2000). Although C had a role in protecting IMR-32 against lytic cell death, no modulation of apoptotic cell death was seen. Lytic, but not sublytic, C attack resulted in loss of CRegs and CD200, the latter an immune modulator, suggesting that one mechanism contributing to phagocyte activation in areas of C-mediated damage could be loss of the tolerogenic CD200 signal, and that C activation may be further enhanced on lysed cell membranes leading to further inflammation. Also of relevance to this work was the finding in Chapter 3 that C opsonisation of normal IMR-32 cells resulted in an increase in the number of cells phagocytosed, implying that healthy neuronal cells may also be removed under inflammatory conditions, resulting in additional neuronal loss.

The observations made in vitro on the initiation of necrosis or apoptosis in neuronal cells, and the effects of C on cells proceeding through apoptosis, provoked studies in vivo using a rat model of excitotoxic neuronal death. The model chosen was based on KA, an excitotoxin that affects neurons predominantly in the hippocampal CA1 and CA3 areas, and was performed on C6 deficient Lewis rats with or without reconstitution with human C6. The results showed that the MAC likely promoted excitotoxin-induced neuronal apoptosis, although clinical scores did not show a significant difference. However, the interaction between MAC and apoptotic neurons appeared more complex than a simply promoting apoptosis: the presence of MAC likely lowered the threshold for seizure activity induced by KA, but once seizures were established, the number and distribution of apoptotic cells seen was

2. Issues raised by thesis findings and future directions

2.1. Use of a neuroblastoma cell line vs primary neuron culture

All of the in vitro work in this thesis was carried out using a human neuroblastoma cell line, since human primary neurons are hard to obtain and handle. This approach has a number of benefits, as the cells are easy to grow in large numbers, and are easy to handle experimentally. The IMR-32 line was specifically chosen, as it possesses similar C activation and control characteristics to primary human fetal neurons (Gasque, Thomas et al. 1996; Singhrao, Neal et al. 2000). However, there are drawbacks to using neuronal cell lines as a model of primary neurons. These cells are actively dividing, whereas primary neurons are post-mitotic, and this may alter their susceptibility to apoptosis induction. This may affect susceptibility to CPT, since this agent preferentially targets cells in S-phase, although it also has cell-cycle independent apoptotic activity (Morris and Geller 1996; Johnson, Ng et al. 1997). As a model for studying neuroblastoma responses to CPT-based chemotherapy, this approach has much to offer, and indeed has been used as such in a collaborative project by myself and others (Donev, Cole et al. in press).

Cell lines may also differ in their susceptibility to C attack, and their recovery and resistance mechanisms, since many tumour cells have increased levels of cell surface CReg, and possess a wide array of other C protection strategies (Jurianz, Ziegler et al. 1999; Jurianz, Ziegler et al. 2001). It is not clear if IMR-32 possess additional resistance mechanisms to primary neuronal cells, but they are clearly susceptible to C-mediated killing, have a similar CReg profile to primary cells. Although many neuroblastoma cell lines express glutamate receptor genes, IMR32 are resistant to excitotoxic cell death (Yoshioka, Ikegaki et al. 1996), a missing link with the in vivo work .

There are several approaches to solving the problem of relevance to primary cells. The ideal approach is to use primary human neurons, although there are ethical issues in using aborted foetal tissue, and availability is limited. Human neuronal stem cells can also be cultured, and will differentiate into mature neurons, and are thus a good alternative, but will not be exactly the same as a mature neuron as the in vitro differentiation is unlikely to fully follow differentiation in vivo. The second approach is to use primary rat neurons. These have the advantage of being readily obtainable, and in my work would support elements of the human in vitro work and provide a link to the in vivo studies in the rat; the work carried out on the rat neuroblastoma cell line also suggests that at least some of the phenomena described are likely to be conserved between species. However, the question always remains of the applicability of the findings to human disease, an issue that affects all animal research.

The final approach uses the NT2 human teratoma cell line differentiated with retinoic acid. This results in a nearly pure (>95%) post-mitotic neuronal population (NT2-N) once the mitotic contaminants have been selectively killed (Pleasure, Page et al. 1992). Although these are not true mature neurons, they do possess embryonic human neurons markers, suggesting that they are a close model (Pleasure, Page et al. 1992). This approach was attempted during the thesis project, and cells were successfully differentiated once; however a problem with mycoplasma infection prevented CReg and apoptosis analysis, and further use of these cells was abandoned.

Whichever approach is used, the aim would be the same: to establish whether the key phenomenon described in this thesis occurs in primary cells. The major finding to be confirmed is CReg loss during apoptosis and whether cells are more readily opsonised following C activation. It would also be important to examine whether induced protection occurred and whether MAC altered apoptosis induction. The mechanisms involved need not be as extensively investigated as in the cell line, since if the primary phenomenon is the same it is likely that the mechanisms involved are also similar. It

would also be possible to study the influence of C activation in modulating excitotoxic cell death using primary cells since they possess intact glutamate receptors. Single cell calcium flux studies and live cell imaging would be useful in examining the effect of MAC on excitotoxin-induced calcium changes and cell death, since MAC has previously been shown to sensitise cells to excitotoxin-induced necrosis, and the mechanisms involved are currently unclear (Xiong and McNamara 2002).

2.2. Neuronal apoptosis and axonal transection

The in vitro studies in this thesis examined the effect of pro-apoptotic stimuli and C activation on the entire neuronal cell. However, in many disease states C activation occurs on axons distant from the neuronal cell body. In ADEAE axonal damage is MAC mediated, and in MS lesions axons are damaged (Trapp, Peterson et al. 1998; Peterson, Bo et al. 2001; Mead, Singhrao et al. 2002), but this occurs in the white matter, and is often far from the neuronal cell body. Evidence suggests that axonal transection can induce neuronal apoptosis, and that these events are closely linked (Peterson, Bo et al. 2001; Berliocchi, Fava et al. 2005). Thus it is possible that lytic MAC attack on axons causing a transection could induce apoptosis in the neuronal cell body, even though I have shown that MAC does not induce neuronal apoptosis when the whole cell is attacked by a sublytic dose of C. This could be studied in more detail using either rat primary neurons or NT2-N cells, with axons induced to grow into a chamber isolated from the cell body, and then attacking the axons with C. Live cell imaging and calcium flux analysis would be useful in assessing the temporal effects of this, and it would be interesting to examine whether sublytic C attack on axons could induce protection to lytic C attack in other parts of the cell.

2.3. Intracellular signalling pathways and gene transcription profiles activated by sublytic C

The mechanism whereby sublytic C causes induced protection from lytic C attack has been investigated in other cell types. This has mainly been investigated in leukaemia cell lines, and

depends upon MAC-induced calcium influx, and involves activation of protein kinase C and ERK (Kraus and Fishelson 2000; Kraus, Seger et al. 2001). Hsp70 also appears to play a role (Fishelson, Hochman et al. 2001), but beyond this little else is known. In chapter 3, I demonstrated that induced protection in neuronal cells was largely MAC-independent, in contrast to published data in leukocytes. The intracellular signalling pathways employed are therefore likely to be different to those activated by MAC. To further characterise this it would first be essential to pinpoint the step in the C cascade where signalling to the cell takes place using various C-depleted sera, and then confirming this using purified C components or antibody cross-linking. There are a number of candidates in the C system that could be responsible for signalling to the cell in this context. The anaphylatoxins C3a or C5a may be responsible, since C5a has been shown to modulate neuronal cell death under other circumstances (Mukherjee and Pasinetti 2001). CD46 has also been shown to be involved in signalling to cells such as macrophages and T-lymphocytes (Kurita-Taniguchi, Fukui et al. 2000; Sanchez, Feito et al. 2004), and C1q has also been shown to transduce signals to cells such as neutrophils (Leigh, Ghebrehiwet et al. 1998). Once the responsible step in the C systems has been identified, it would be possible to examine the signalling pathway in more detail. Immunoprecipitation to pull down associated molecules could be used, in addition to Western blots for phosphorylated proteins such as ERK1/2 and those involved in other pathways, and EMSA used to identify the transcription factors involved. The use of specific signalling pathway inhibitors would likely create problems since many of these are irreversible, and thus would also affect the subsequent lytic C attack, making the data difficult to interpret. 2D DIGE and gene expression profiling could also be used to identify novel protein targets, which would then be verified using Western blotting and real-time PCR.

Analysis of other cell types would be useful in order to identify common signalling pathways via MAC and other C activation products, since at present a number of different pathways have been

implicated, but no unifying concept has emerged. Such data may provide useful information for future therapeutic design, particularly in targeting mechanisms that either increase resistance to C-mediated damage, where C activation in uncontrolled and deleterious, or in sensitising cells that are surviving in the face of C attack inappropriately, as in cancer.

2.4. Functions and consequences of CReg and CD200 shed during apoptosis and necrosis

Neuronal cell death via apoptosis results in the loss of membrane-bound CRegs, shed into the environment by either MMP-mediated cleavage (CD46) or on apoptotic blebs or as a soluble form (CD59). A recent study from this department showed that CD46 is also shed from apoptotic Jurkat cells (Elward, Griffiths et al. 2005). This was noted to be dependent on caspases, as in my studies, but differed in that caspase-3 was found not to be essential in loss of CD46 from apoptotic Jurkat cells. Given that caspase-3 is central to neuronal apoptosis (Yakovlev and Faden 2001), this perhaps is not surprising, but does indicate that loss of CD46 is dependent on different pathways in different cells.

Apoptotic Jurkat were also more readily opsonised by C activation products, a finding that correlated with CD46 loss (Elward, Griffiths et al. 2005), as in my study, indicating that under inflammatory conditions C-mediated clearance may become increasingly important. However, MAC deposition was not significant in the Jurkat model, suggesting that lysis of apoptotic neurons may be cell specific. Interestingly, C1q binding was shown to be dependent on nucleic acid that had translocated to the cell surface and detected as a PI^{dim} population on flow cytometry. I also observed a PI^{dim} population before PI^{high} cells were observed, but only for UV treated cells that had already become Annexin-V-FITC positive. On most apoptotic cells C1q binding to DNA may be important in order to activate C via the classical pathway, and in Jurkat this appeared to be the critical factor in opsonisation with C, since both PI^{dim}/Ann- and PI^{dim}/Ann+ cells activated C to the same degree. The importance of CD46 loss from apoptotic cells in promoting C activation was thus not

clear from the studies in Jurkat, since CD46 was lost only from PI^{dim}/Ann+ cells. Neuronal cells spontaneously activate C via the classical pathway, possibly making C activation via exposed nucleic acids redundant on apoptotic neurons; indeed, I noted no difference in C1q binding between control and apoptotic IMR-32. These cells may therefore rely more heavily on CD46 loss to increase opsonisation and flagging for phagocytosis than other cell types.

Notably, it is not just apoptotic cells that shed CReg: necrotic cells also lose these molecules. I have shown that C-mediated lysis results in loss of CReg from apoptotic neurons, and H_2O_2 treatment of Jurkat cells results in the same phenomenon (Elward, Griffiths et al. 2005). In the latter study, CRegs were shed in a soluble, not bleb-associated, form and this is likely to be true for necrotic neurons also. Since both apoptotic and necrotic cells shed CReg, the function of this event thus becomes important: do the soluble CRegs remain active, and are apoptotic blebs protected from C-mediated lysis by the CD59 on their surface? Data from tumour cells that constitutively shed soluble CD46 via an MMP-mediated mechanism shows that the molecule is functionally active (Hakulinen, Junnikkala et al. 2004), and CD59 has been shown to protect exosomes from C attack (Clayton, Harris et al. 2003). This suggests that the shed CReg from apoptotic neuronal cells should also be active in controlling C activation and lysis, but this remains to be demonstrated.

The placing of C1q and CD46 on apoptotic blebs has been suggested to modulate immune responses, acting as an "appetiser" signal to phagocytic cells prior to ingestion of the apoptotic cell body (Elward, Griffiths et al. 2005). It is possible that CD46 shed in a soluble form may also have such a function, although this remains to be shown. CD59 was also present on apoptotic blebs in my model, but was not shed from apoptotic Jurkat. The significance of this difference is not known, but apoptotic neurons shed CD59 early in apoptosis, well before cell surface changes were noted, implying that increased synthesis was taking place and therefore that this was important to the fate of

the apoptotic neuron. It is possible, given the increased capacity of apoptotic neurons to activate C right through to MAC formation and their loss of CD46, that CD59 becomes the major CReg protecting against lysis, and that increased synthesis allows protection to be passed to apoptotic blebs while also maintaining CD59 levels on the cell body. Other apoptotic cell types may not require this, as they may not spontaneously activate C through to the terminal pathway. Interestingly, early apoptotic Jurkat have been shown to be more susceptible to C-mediated lysis than control cells (Attali, Gancz et al. 2004), but in this case the cells were sensitised with antibody prior to C attack, implying that under inflammatory conditions the outcome of C activation on apoptotic lymphocytes may be very different, and approximate more readily to the findings in apoptotic neurons.

CD59 and CD46 have also been shown to have C-independent actions, and it is possible that molecules shed from apoptotic cells also have further functions. CD59 has been shown to induce proliferation in fibroblasts and other cell types, and appears to have a function in modulating T-cell mediated immune responses (Longhi, Sivasankar et al. 2005). Preliminary data from this laboratory also shows that CD59 binds to primary human neutrophils, monocytes, and macrophages, suggesting that it may modulate the functions of these cell types, possibly influencing phagocytosis. This would be a complementary role to that of CD200, shed and regulated in a similar fashion to CD59 under these circumstances, which is known to modulate myeloid cell activation (Hoek, Ruuls et al. 2000). The loss of CD200 from apoptotic cells may function to activate microglia and other phagocytes in the immediate vicinity, in order to remove debris efficiently. Interestingly, this finding is in direct contrast to the findings in apoptotic dendritic cells, where CD200 expression was increased as a result of p53 activation, suggesting a dampening of phagocyte activity (Rosenblum, Olasz et al. 2004). This has been proposed to result in tolerance to the shed antigens; in support of this experimental autoimmune uveoretinitis in rats was shown to be more aggressive when the CD200 receptor was blocked (Banerjee and Dick 2004). Thus it is possible that phagocyte responses may be modulated by

the presence of soluble CD200 and CD59, which may reduce activation and immune reactivity, and promote repair by inducing proliferation in glial cells and fibroblasts. These hypotheses could be tested using the phagocytosis assay I have developed, and examining the influence of these molecules on phagocytosis itself and on the cytokines secreted by the macrophages. It would also be important to examine the effect of soluble CD59 on astrocyte proliferation and activation, since these cells are key in gliosis following CNS injury.

2.5. C activation in MS: a dual role?

It is well established that MAC causes damage in neuroinflammatory diseases such as MS, particularly in the acute phase of pattern II (antibody and C-mediated) disease (Kornek and Lassmann 2003; Pittock, McClelland et al. 2005) and its animal model ADEAE (Mead, Singhrao et al. 2002). Under these circumstances, neurons are exposed to high levels of cellular stress, and C activation is high. My data suggests that when susceptible neurons undergo apoptosis acutely they are more vulnerable to C-mediated killing, and exposure to lytic C attack may cause further damage. In addition, in acute insults, the glial cell response takes time (Campbell, Finlay et al. 2004), and cellular responses that may help limit damage by clearing debris are therefore delayed.

Evidence is accumulating that C and the MAC have important roles in protecting cells such as oligodendrocytes from apoptosis (Soane, Rus et al. 1999; Dashiell, Rus et al. 2000), and in promoting repair during the recovery phase of disease (Weerth, Rus et al. 2003). Under chronic inflammatory conditions, or during the recovery phase when inflammation is beginning to resolve, sublytic levels of C activation are more likely to occur. My work has shown that this helps to protect neurons from lytic C attack, thus promoting recovery and repair by preserving the surviving population and helping to limit further inflammation. Data from CPT treated cells also suggests that cells surviving a pro-apoptotic insult are more resistant to C-mediated killing, again limiting damage. Thus a number of

different mechanisms appear to be at play once the inflammatory response falls below a certain threshold.

We can now appreciate a dual role for C, and in particular the MAC, first in its classical role as being the perpetrator of damage in pattern II MS in the acute setting, but then switching to a role that promotes cell survival and repair in the recovery phase. Under chronic inflammatory conditions, these opposing roles are likely to be finely balanced, with perhaps only small stimuli changing the balance and causing a disease flare and progression of disability. This dichotomy is evident in my work, since the kinetics and outcomes of apoptotic neuronal death also appear to have an acute versus chronic split, with cells undergoing apoptosis soon after the apoptotic challenge being more pro-inflammatory and vulnerable to C-mediated killing, compared to cells where apoptosis occurs later that are more resistant to killing.

In the in vivo model of neuronal death in my thesis, only the acute consequences of excitotoxin exposure were investigated. A further study would be required to examine whether C and MAC influenced repair processes, either using the same kainic acid model to specifically examine neuronal death, or EAE to look at MS-related pathology as a whole, but looking at longer time points, for example 3 days, 10 days, one month and three months. Analysis of cellular infiltrate, extent of axonal loss, and clinical neurological deficits would be instructive, as would analysis of apoptosis and cell cycle genes. Additional animal models, such as for AD or other chronic inflammatory models could be used to establish whether C activation and the MAC were detrimental or advantageous in these contexts.

2.6. C mediated clearance of debris and role in AD

C is known to be activated in AD, where components of the classical pathway and C activation

products are found associated with amyloid plaques (Afagh, Cummings et al. 1996; Emmerling, Watson et al. 2000). Opsonisation of β-amyloid with C is critical in removing this toxic peptide from the brain, since inhibition of C by the over-expression of soluble Crry (sCrry) in a transgenic mouse expressing human amyloid precursor protein (hAPP) resulted in marked worsening of disease with plaque accumulation and neuronal degeneration (Wyss-Coray, Yan et al. 2002). Thus C is important in clearing potentially toxic proteinatious debris under pathological conditions in vivo, a function that is likely critical in preventing disease progression, since clearance of amyloid using β-amyloid peptide immunisation has been shown to result in improved clinical outcomes in mouse models of AD (Janus, Pearson et al. 2000; Morgan, Diamond et al. 2000). My studies have suggested an additional role for C in clearing neuronal apoptotic debris, principally via the loss of CD46 and subsequent increased C3b opsonisation and phagocytosis. Neuronal cells activate C via the classical pathway, and C1q had been found associated with neurons in AD brains, but not control brains, suggesting either that the neurons are synthesising C1q, or that C1q is bound to their cell surface (Afagh, Cummings et al. 1996), indicating that the relevant components are present for opsonisation to occur. Thus C activation may be seen as a useful protective response to potentially toxic material in the brain.

Despite this, C also has a role in promoting neuronal degeneration in AD. The presence of β-amyloid and neurofibrillary tangles in the brain does not necessarily imply that dementia is present and the patient has AD, since some patients with large numbers of plaques and tangles at post-mortem were clinically normal during life (Lue, Brachova et al. 1996). The additional factor in causing neuronal loss and clinical dementia in AD is inflammation, and the extent of MAC deposition correlates well with loss of neuronal synapses, a good marker of disease severity (Lue, Brachova et al. 1996). Indeed, neurons in AD may be particularly susceptible to MAC-mediated damage, since CD59 levels have been found to be reduced in areas of active pathology in AD, and β-amyloid has been shown to

activate C and cause MAC-mediated damage to neuronal cells in vitro in the absence of an exogenous source of C when CD59 is compromised (Shen, Sullivan et al. 1998). However, others have found increased levels of CD59 where MAC staining was high in AD brains, a contradictory finding indicating that the precise expression pattern and role of CD59 in AD is still unclear. The differences may reflect the stage of disease and whether neurons are actively undergoing apoptosis and thus shedding CD59, or have survived a pro-apoptotic insult and have higher CD59 levels than normal, as suggested by my work.

In AD, neurons likely defend themselves from MAC attack by removing the lesions from the cell surface by internalisation (Itagaki, Akiyama et al. 1994), a mechanism also utilised by other cell types (Morgan, Dankert et al. 1987). My studies have confirmed that neuronal cells are capable of recovering from MAC attack as indicated by restoration of intracellular calcium levels following sublytic attack, thus supporting the hypothesis that the internalised MAC in neurons in AD brain may be recovering, and not simply defending themselves during a pre-lytic phase of C-mediated cell death. The mechanism by which neuronal cells recover in my model was not further investigated, although live cell imaging using fluorescence labelled C9 in reactive lysis would likely provide an answer. I also demonstrated that protective responses in neurons to lytic MAC may be enhanced by sublytic C attack, a finding that may also be of relevance in AD, since it may allow neurons to survive longer under pathological conditions. This may be of benefit in reducing neuronal loss and therefore neurological deficit, but keeping diseased neurons alive rather than disposing of them via apoptosis may be inappropriate if they continue to process APP abnormally or act as a nidus for C activation and inflammation. The details of the mechanisms responsible for "induced protection" in neurons are unknown, but they may improve the efficiency of the recovery observed using calcium flux studies. This could be further studied by incubating IMR-32 with C8d serum to induce protection, and then attacking with reactive lysis while monitoring calcium flux using the assays I

have set up, or following MAC fate with live cell imaging. The recovery kinetics would then give important clues as whether induced protection involves modulating these processes. Indeed, similar studies on IMR-32 with NFTs or producing toxic β-amyloid may also provide clues as to the mechanisms leading to cell death under pathological conditions, and whether diseased neurons have the capacity to effectively defend themselves during C attack.

2.7. Therapeutic potential of MMP-inhibitors in C-mediated CNS disease

I have shown that MMP-3, -8 and -9 are involved in the cleavage of CD46 from apoptotic neuronal cells, which leads to increased C activation and increased C-mediated lysis. Importantly, these effects can be blocked by MMP-inhibition in vitro, and this suggests that they may have a role in controlling pathological C activation in vivo. Indeed, C activation was noted on apoptotic neurons in the KA model of neuronal cell death, and the in vitro data suggest that such inhibitors may be useful in specifically protecting apoptotic neurons against C activation and lysis, since MMP-inhibitors had no protective effect on susceptibility of viable cells to lysis.

MMPs are known to be up-regulated and active in neuroinflammatory diseases such as MS (Avolio, Ruggieri et al. 2003), and have been suggested as biomarkers of neuroinflammation (Rosenberg 2005). MMPs have also been shown to play a pivotal role in various animal models of disease, including KA induced neurodegeneration and in EAE (Hewson, Smith et al. 1995; Penkowa, Florit et al. 2005). MMP-3 and MMP-9 appear to be the most important (Jourquin, Tremblay et al. 2003), but the role of MMP-8 has not been fully addressed. Importantly, MMP inhibition is neuroprotective in many of these animal models, an effect that is attributed to reduced BBB permeability, migration of immune cells, and modified extracellular matrix (Rosenberg 2002). The role of MMP inhibitors in controlling C activation has not been addressed, and in the KA model used in my thesis could be easily assessed. It would also be useful to investigate the role of C activation in general in

conjunction with studies of MMP related C activation changes, perhaps by using a Crry-Fc fusion protein, since MMP inhibitors are already known to be neuroprotective in this model. Such an approach would help to distinguish between effects of MMP inhibition on C activation, and C-independent MMP effects. This could also be done using MMP inhibitors in KA treated C3 deficient mice, which would perhaps be a better approach in unravelling these various roles.

3. Conclusion

Apoptosis and C activation are important processes in neurological disease. C activation has long been seen as detrimental, but has important roles in clearing apoptotic and proteinacious debris and may also promote cell survival. In this thesis, I have shown that C is important in modulating neuronal cell turnover, both by inducing lytic cell death and possibly in facilitating excitotoxin-induced seizures, but also promoting cell survival when present in sublytic doses. C is more readily activated on apoptotic neuronal cells due to loss of CD46, and the resulting opsonisation targets them for removal by phagocytosis. However, CReg loss also leaves them more vulnerable to C-mediated lysis, which may worsen inflammation if the cell is not disposed of efficiently. MMP inhibition reduces CD46 loss and protects apoptotic neuronal cells from lysis.

These data have revealed important mechanisms whereby sublytic C protects neuronal cells, and have unravelled the altered handling of C activation during neuronal apoptosis. This work suggests that C has a dual role in pathology, where C activation in the acute phase is detrimental and causes damage, but in the recovery phase aids repair. This raises questions as to the appropriate use of C therapeutics: from my conclusions, this would only be beneficial during the acute phase of illness, and should target MAC, since other arms of the C system are beneficial in removing cellular corpses, and in

protecting against lytic cell death and apoptosis.

References:

Acosta, J., Hettinga, J., Fluckiger, R., Krumrei, N., Goldfine, A., Angarita, L. and Halperin, J. (2000). "Molecular basis for a link between complement and the vascular complications of diabetes." Proc Natl Acad Sci U S A **97**(10): 5450-5455.

Afagh, A., Cummings, B. J., Cribbs, D. H., Cotman, C. W. and Tenner, A. J. (1996). "Localization and cell association of C1q in Alzheimer's disease brain." Exp Neurol **138**(1): 22-32.

Ahmed, Z., Doward, A. I., Pryce, G., Taylor, D. L., Pocock, J. M., Leonard, J. P., Baker, D. and Cuzner, M. L. (2002). "A role for caspase-1 and -3 in the pathology of experimental allergic encephalomyelitis : inflammation versus degeneration." Am J Pathol **161**(5): 1577-1586.

Attali, G., Gancz, D. and Fishelson, Z. (2004). "Increased sensitivity of early apoptotic cells to complement-mediated lysis." Eur J Immunol **34**(11): 3236-3245.

Avolio, C., Ruggieri, M., Giuliani, F., Liuzzi, G. M., Leante, R., Riccio, P., Livrea, P. and Trojano, M. (2003). "Serum MMP-2 and MMP-9 are elevated in different multiple sclerosis subtypes." J Neuroimmunol **136**(1-2): 46-53.

Banerjee, D. and Dick, A. D. (2004). "Blocking CD200-CD200 receptor axis augments NOS-2 expression and aggravates experimental autoimmune uveoretinitis in Lewis rats." Ocul Immunol Inflamm **12**(2): 115-125.

Barrington, R., Zhang, M., Fischer, M. and Carroll, M. C. (2001). "The role of complement in inflammation and adaptive immunity." Immunol Rev **180**: 5-15.

Bell, D. A. and Morrison, B. (1991). "The spontaneous apoptotic cell death of normal human lymphocytes in vitro: the release of, and immunoproliferative response to, nucleosomes in vitro." Clin Immunol Immunopathol **60**(1): 13-26.

Benzaquen, L. R., Nicholson-Weller, A. and Halperin, J. A. (1994). "Terminal complement proteins C5b-9 release basic fibroblast growth factor and platelet-derived growth factor from

endothelial cells." Journal of Experimental Medicine **179**(3): 985-992.

Berliocchi, L., Fava, E., Leist, M., Horvat, V., Dinsdale, D., Read, D. and Nicotera, P. (2005). "Botulinum neurotoxin C initiates two different programs for neurite degeneration and neuronal apoptosis." J Cell Biol **168**(4): 607-618.

Bhakdi, S., Kuller, G., Muhly, M., Fromm, S., Seibert, G. and Parrisius, J. (1987). "Formation of transmural complement pores in serum-sensitive Escherichia coli." Infect Immun **55**(1): 206-210.

Bhakdi, S. and Tranum-Jensen, J. (1991). "Complement lysis: a hole is a hole." Immunol Today **12** (9): 318-320; discussion 321.

Bickerstaff, M. C., Botto, M., Hutchinson, W. L., Herbert, J., Tennent, G. A., Bybee, A., Mitchell, D. A., Cook, H. T., Butler, P. J., Walport, M. J. and Pepys, M. B. (1999). "Serum amyloid P component controls chromatin degradation and prevents antinuclear autoimmunity." Nat Med **5**(6): 694-697.

Bjartmar, C., Wujek, J. R. and Trapp, B. D. (2003). "Axonal loss in the pathology of MS: consequences for understanding the progressive phase of the disease." J Neurol Sci **206**(2): 165-171.

Bordet, J. (1900). "Les serums haemolytiques, leurs antitoxines et les therories des serum cytolytiques." Annals de Institute Pasteur **15**: 257-270.

Born, J. and Bhakdi, S. (1986). "Does complement kill E. coli by producing transmural pores?" Immunology **59**(1): 139-145.

Botto, M. (2001). "Links between complement deficiency and apoptosis." Arthritis Res **3**(4): 207-210.

Botto, M., Dell'Agnola, C., Bygrave, A. E., Thompson, E. M., Cook, H. T., Petry, F., Loos, M., Pandolfi, P. P. and Walport, M. J. (1998). "Homozygous C1q deficiency causes glomerulonephritis associated with multiple apoptotic bodies." Nat Genet **19**(1): 56-59.

Brandt, J., Pippin, J., Schulze, M., Hansch, G. M., Alpers, C. E., Johnson, R. J., Gordon, K. and Couser, W. G. (1996). "Role of the complement membrane attack complex (C5b-9) in mediating experimental mesangioproliferative glomerulonephritis." Kidney International **49** (2): 335-343.

Bratton, D. L., Fadok, V. A., Richter, D. A., Kailey, J. M., Guthrie, L. A. and Henson, P. M. (1997). "Appearance of phosphatidylserine on apoptotic cells requires calcium-mediated nonspecific flip-flop and is enhanced by loss of the aminophospholipid translocase." J Biol Chem **272**(42): 26159-26165.

Brown, S., Heinisch, I., Ross, E., Shaw, K., Buckley, C. D. and Savill, J. (2002). "Apoptosis disables CD31-mediated cell detachment from phagocytes promoting binding and engulfment." Nature **418**(6894): 200-203.

Campbell, A. K., Daw, R. A., Hallett, M. B. and 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**(2): 551-560.

Campbell, A. K., Patel, A., Houston, W. A., Scolding, N. J., Frith, S., Morgan, B. P. and Compston, D. A. (1989). "Photoproteins as indicators of intracellular free Ca2+." J Biolumin Chemilumin **4**(1): 463-474.

Campbell, S. J., Finlay, M., Clements, J. M., Wells, G., Miller, K. M., Perry, V. H. and Anthony, D. C. (2004). "Reduction of excitotoxicity and associated leukocyte recruitment by a broad-spectrum matrix metalloproteinase inhibitor." J Neurochem **89**(6): 1378-1386.

Carney, D. F., Koski, C. L. and Shin, M. L. (1985). "Elimination of terminal complement intermediates from the plasma membrane of nucleated cells: the rate of disappearance differs for cells carrying C5b-7 or C5b-8 or a mixture of C5b-8 with a limited number of C5b-9." J Immunol **134**(3): 1804-1809.

Cid, C., Alvarez-Cermeno, J. C., Regidor, I., Plaza, J., Salinas, M. and Alcazar, A. (2003). "Caspase

inhibitors protect against neuronal apoptosis induced by cerebrospinal fluid from multiple sclerosis patients." J Neuroimmunol **136**(1-2): 119-124.

Clayton, A., Harris, C. L., Court, J., Mason, M. D. and Morgan, B. P. (2003). "Antigen-presenting cell exosomes are protected from complement-mediated lysis by expression of CD55 and CD59." Eur J Immunol **33**(2): 522-531.

Clements, J. M., Cossins, J. A., Wells, G. M., Corkill, D. J., Helfrich, K., Wood, L. M., Pigott, R., Stabler, G., Ward, G. A., Gearing, A. J. and Miller, K. M. (1997). "Matrix metalloproteinase expression during experimental autoimmune encephalomyelitis and effects of a combined matrix metalloproteinase and tumour necrosis factor-alpha inhibitor." J Neuroimmunol **74**(1-2): 85-94.

Compston, D. A., Morgan, B. P., Campbell, A. K., Wilkins, P., Cole, G., Thomas, N. D. and Jasani, B. (1989). "Immunocytochemical localization of the terminal complement complex in multiple sclerosis." Neuropathol Appl Neurobiol **15**(4): 307-316.

Couser, W. G., Pippin, J. W. and Shankland, S. J. (2001). "Complement (C5b-9) induces DNA synthesis in rat mesangial cells in vitro." Kidney International **59**(3): 905-912.

Cragg, M. S., Howatt, W. J., Bloodworth, L., Anderson, V. A., Morgan, B. P. and Glennie, M. J. (2000). "Complement mediated cell death is associated with DNA fragmentation." Cell Death Differ **7**(1): 48-58.

Cybulsky, A. V., Bonventre, J. V., Quigg, R. J., Lieberthal, W. and Salant, D. J. (1990). "Cytosolic calcium and protein kinase C reduce complement-mediated glomerular epithelial injury." Kidney Int **38**(5): 803-811.

Cybulsky, A. V., Takano, T., Papillon, J. and McTavish, A. J. (1999). "Complement C5b-9 induces receptor tyrosine kinase transactivation in glomerular epithelial cells." Am J Pathol **155**(5): 1701-1711.

Daemen, M. A., van 't Veer, C., Denecker, G., Heemskerk, V. H., Wolfs, T. G., Clauss, M.,

Vandenabeele, P. and Buurman, W. A. (1999). "Inhibition of apoptosis induced by ischemia-reperfusion prevents inflammation." J Clin Invest **104**(5): 541-549.

D'Ambrosio, A. L., Pinsky, D. J. and Connolly, E. S. (2001). "The role of the complement cascade in ischemia/reperfusion injury: implications for neuroprotection." Mol Med 7(6): 367-382.

Dashiell, S. M., Rus, H. and Koski, C. L. (2000). "Terminal complement complexes concomitantly stimulate proliferation and rescue of Schwann cells from apoptosis." Glia **30**(2): 187-198.

Davie, C. A., Barker, G. J., Webb, S., Tofts, P. S., Thompson, A. J., Harding, A. E., McDonald, W. I. and Miller, D. H. (1995). "Persistent functional deficit in multiple sclerosis and autosomal dominant cerebellar ataxia is associated with axon loss." Brain **118 (Pt 6)**: 1583-1592.

De Simone, R., Ajmone-Cat, M. A. and Minghetti, L. (2004). "Atypical antiinflammatory activation of microglia induced by apoptotic neurons: possible role of phosphatidylserine-phosphatidylserine receptor interaction." Mol Neurobiol **29**(2): 197-212.

deCathelineau, A. M. and Henson, P. M. (2003). "The final step in programmed cell death: phagocytes carry apoptotic cells to the grave." Essays Biochem **39**: 105-117.

Dessauer, A., Rother, U. and Rother, K. (1984). "Freeze-thaw activation of the complement attack phase: I. Separation of two steps in the formation of the active C--56 complex." Acta Pathol Microbiol Immunol Scand Suppl **284**: 75-81.

Dessauer, A., Rother, U. and Rother, K. (1984). "Freeze-thaw activation of the complement attack phase: II. Comparison of convertase generated C--56 with C--56 generated by freezing and thawing." Acta Pathol Microbiol Immunol Scand Suppl **284**: 83-88.

Donev, R., Cole, D. S., Baalsubramanian, S., Hughes, T. R. and Morgan, B. P. (in press). "p53 regulates cellular resistance to complement lysis through enhanced expression of CD59." Cancer Research.

Dowling, P., Husar, W., Menonna, J., Donnenfeld, H., Cook, S. and Sidhu, M. (1997). "Cell death and birth in multiple sclerosis brain." J Neurol Sci **149**(1): 1-11.

Earnshaw, W. C., Martins, L. M. and Kaufmann, S. H. (1999). "Mammalian caspases: structure, activation, substrates, and functions during apoptosis." Annu Rev Biochem **68**: 383-424.

Ehrlich, P. and Morgenroth, J. (1899). "Zur theorie der lysinwirkung." Berlin Klin. Wochenchr. **36**: 6-9.

Elward, K. and Gasque, P. (2003). ""Eat me" and "don't eat me" signals govern the innate immune response and tissue repair in the CNS: emphasis on the critical role of the complement system." Mol Immunol **40**(2-4): 85-94.

Elward, K., Griffiths, M., Mizuno, M., Harris, C. L., Neal, J. W., Morgan, B. P. and Gasque, P. (2005). "CD46 plays a key role in tailoring innate immune recognition of apoptotic and necrotic cells." J Biol Chem **280**(43): 36342-36354.

Emmerling, M. R., Watson, M. D., Raby, C. A. and Spiegel, K. (2000). "The role of complement in Alzheimer's disease pathology." Biochim Biophys Acta **1502**(1): 158-171.

Enari, M., Sakahira, H., Yokoyama, H., Okawa, K., Iwamatsu, A. and Nagata, S. (1998). "A caspase-activated DNase that degrades DNA during apoptosis, and its inhibitor ICAD." Nature **391** (6662): 43-50.

Esser, A. F. (1991). "Big MAC attack: complement proteins cause leaky patches." Immunol Today **12** (9): 316-318; discussion 321.

Fadok, V. A., Bratton, D. L., Konowal, A., Freed, P. W., Westcott, J. Y. and Henson, P. M. (1998). "Macrophages that have ingested apoptotic cells in vitro inhibit proinflammatory cytokine production through autocrine/paracrine mechanisms involving TGF-beta, PGE2, and PAF." J Clin Invest **101**(4): 890-898.

Fadok, V. A., Voelker, D. R., Campbell, P. A., Cohen, J. J., Bratton, D. L. and Henson, P. M. (1992). "Exposure of phosphatidylserine on the surface of apoptotic lymphocytes triggers specific recognition and removal by macrophages." J Immunol **148**(7): 2207-2216.

Farkas, I., Baranyi, L., Liposits, Z. S., Yamamoto, T. and Okada, H. (1998). "Complement C5a

anaphylatoxin fragment causes apoptosis in TGW neuroblastoma cells." Neuroscience **86**(3): 903-911.

Fawcett, J. W. R., A.E. Dunnett, S.B. (2001). Brain damage, brain repair. Oxford, Oxford University Press.

Ffrench-Constant, C. (1994). "Pathogenesis of multiple sclerosis." Lancet **343**(8892): 271-275.

Fishelson, Z., Attali, G. and Mevorach, D. (2001). "Complement and apoptosis." Mol Immunol **38**(2-3): 207-219.

Fishelson, Z., Hochman, I., Greene, L. E. and Eisenberg, E. (2001). "Contribution of heat shock proteins to cell protection from complement-mediated lysis." Int Immunol **13**(8): 983-991.

Fujikawa, D. G., Shinmei, S. S. and Cai, B. (2000). "Kainic acid-induced seizures produce necrotic, not apoptotic, neurons with internucleosomal DNA cleavage: implications for programmed cell death mechanisms." Neuroscience **98**(1): 41-53.

Gaipl, U. S., Kuenkele, S., Voll, R. E., Beyer, T. D., Kolowos, W., Heyder, P., Kalden, J. R. and Herrmann, M. (2001). "Complement binding is an early feature of necrotic and a rather late event during apoptotic cell death." Cell Death Differ **8**(4): 327-334.

Gasque, P., Fontaine, M. and Morgan, B. P. (1995). "Complement expression in human brain. Biosynthesis of terminal pathway components and regulators in human glial cells and cell lines." J Immunol **154**(9): 4726-4733.

Gasque, P., Ischenko, A., Legoedec, J., Mauger, C., Schouft, M. T. and Fontaine, M. (1993). "Expression of the complement classical pathway by human glioma in culture. A model for complement expression by nerve cells." J Biol Chem **268**(33): 25068-25074.

Gasque, P., Julen, N., Ischenko, A. M., Picot, C., Mauger, C., Chauzy, C., Ripoche, J. and Fontaine, M. (1992). "Expression of complement components of the alternative pathway by glioma cell lines." J Immunol **149**(4): 1381-1387.

Gasque, P., Thomas, A., Fontaine, M. and Morgan, B. P. (1996). "Complement activation on human

neuroblastoma cell lines in vitro: route of activation and expression of functional complement regulatory proteins." J Neuroimmunol **66**(1-2): 29-40.

Gershov, D., Kim, S., Brot, N. and Elkon, K. B. (2000). "C-Reactive protein binds to apoptotic cells, protects the cells from assembly of the terminal complement components, and sustains an antiinflammatory innate immune response: implications for systemic autoimmunity." J Exp Med **192**(9): 1353-1364.

Giambernardi, T. A., Sakaguchi, A. Y., Gluhak, J., Pavlin, D., Troyer, D. A., Das, G., Rodeck, U. and Klebe, R. J. (2001). "Neutrophil collagenase (MMP-8) is expressed during early development in neural crest cells as well as in adult melanoma cells." Matrix Biol **20**(8): 577-587.

Gilliams-Francis, K. L., Quaye, A. A. and Naegele, J. R. (2003). "PARP cleavage, DNA fragmentation, and pyknosis during excitotoxin-induced neuronal death." Exp Neurol **184**(1): 359-372.

Goldsmith, S. K., Wals, P., Rozovsky, I., Morgan, T. E. and Finch, C. E. (1997). "Kainic acid and decorticating lesions stimulate the synthesis of C1q protein in adult rat brain." J Neurochem **68**(5): 2046-2052.

Guo, R. F., Huber-Lang, M., Wang, X., Sarma, V., Padgaonkar, V. A., Craig, R. A., Riedemann, N. C., McClintock, S. D., Hlaing, T., Shi, M. M. and Ward, P. A. (2000). "Protective effects of anti-C5a in sepsis-induced thymocyte apoptosis." J Clin Invest **106**(10): 1271-1280.

Hakulinen, J., Junnikkala, S., Sorsa, T. and Meri, S. (2004). "Complement inhibitor membrane cofactor protein (MCP; CD46) is constitutively shed from cancer cell membranes in vesicles and converted by a metalloproteinase to a functionally active soluble form." Eur J Immunol **34**(9): 2620-2629.

Halperin, J. A., Taratuska, A. and Nicholson-Weller, A. (1993). "Terminal complement complex C5b-9 stimulates mitogenesis in 3T3 cells." Journal of Clinical Investigation **91**(5): 1974-1978.

Hammer, C. H., Hansch, G., Gresham, H. D. and Shin, M. L. (1983). "Activation of the fifth and sixth components of the human complement system: C6-dependent cleavage of C5 in acid and the formation of a bimolecular lytic complex, C5b,6a." J Immunol **131**(2): 892-898.

Hansch, G. M., Seitz, M., Martinotti, G., Betz, M., Rauterberg, E. W. and Gemsa, D. (1984). "Macrophages release arachidonic acid, prostaglandin E2, and thromboxane in response to late complement components." J Immunol **133**(4): 2145-2150.

Hewson, A. K., Smith, T., Leonard, J. P. and Cuzner, M. L. (1995). "Suppression of experimental allergic encephalomyelitis in the Lewis rat by the matrix metalloproteinase inhibitor Ro31-9790." Inflamm Res **44**(8): 345-349.

Hila, S., Soane, L. and Koski, C. L. (2001). "Sublytic C5b-9-stimulated Schwann cell survival through PI 3-kinase-mediated phosphorylation of BAD." Glia **36**(1): 58-67.

Hobom, M., Storch, M. K., Weissert, R., Maier, K., Radhakrishnan, A., Kramer, B., Bahr, M. and Diem, R. (2004). "Mechanisms and time course of neuronal degeneration in experimental autoimmune encephalomyelitis." Brain Pathol **14**(2): 148-157.

Hoek, R. M., Ruuls, S. R., Murphy, C. A., Wright, G. J., Goddard, R., Zurawski, S. M., Blom, B., Homola, M. E., Streit, W. J., Brown, M. H., Barclay, A. N. and Sedgwick, J. D. (2000). "Down-regulation of the macrophage lineage through interaction with OX2 (CD200)." Science **290**(5497): 1768-1771.

Hopkins, J. I., Jones, J. and Morgan, B. P. (1998). "Non-lethal effects of perforin on polymorphonuclear leukocytes." Biochem Soc Trans **26**(1): S50.

Huang, J., Kim, L. J., Mealey, R., Marsh, H. C., Jr., Zhang, Y., Tenner, A. J., Connolly, E. S., Jr. and Pinsky, D. J. (1999). "Neuronal protection in stroke by an sLex-glycosylated complement inhibitory protein." Science **285**(5427): 595-599.

Huber-Lang, M., Sarma, V. J., Lu, K. T., McGuire, S. R., Padgaonkar, V. A., Guo, R. F., Younkin, E. M., Kunkel, R. G., Ding, J., Erickson, R., Curnutte, J. T. and Ward, P. A. (2001). "Role of

C5a in multiorgan failure during sepsis." J Immunol **166**(2): 1193-1199.

Huber-Lang, M. S., Sarma, J. V., McGuire, S. R., Lu, K. T., Guo, R. F., Padgaonkar, V. A., Younkin, E. M., Laudes, I. J., Riedemann, N. C., Younger, J. G. and Ward, P. A. (2001). "Protective effects of anti-C5a peptide antibodies in experimental sepsis." Faseb J **15**(3): 568-570.

Hubschmann, M. V., Skladchikova, G., Bock, E. and Berezin, V. (2005). "Neural cell adhesion molecule function is regulated by metalloproteinase-mediated ectodomain release." J Neurosci Res **80**(6): 826-837.

Hughes, J., Nangaku, M., Alpers, C. E., Shankland, S. J., Couser, W. G. and Johnson, R. J. (2000). "C5b-9 membrane attack complex mediates endothelial cell apoptosis in experimental glomerulonephritis." Am J Physiol Renal Physiol **278**(5): F747-757.

Hynd, M. R., Scott, H. L. and Dodd, P. R. (2004). "Glutamate-mediated excitotoxicity and neurodegeneration in Alzheimer's disease." Neurochem Int **45**(5): 583-595.

Ino, H. and Chiba, T. (2001). "Cyclin-dependent kinase 4 and cyclin D1 are required for excitotoxin-induced neuronal cell death in vivo." J Neurosci **21**(16): 6086-6094.

Itagaki, S., Akiyama, H., Saito, H. and McGeer, P. L. (1994). "Ultrastructural localization of complement membrane attack complex (MAC)-like immunoreactivity in brains of patients with Alzheimer's disease." Brain Res **645**(1-2): 78-84.

Janus, C., Pearson, J., McLaurin, J., Mathews, P. M., Jiang, Y., Schmidt, S. D., Chishti, M. A., Horne, P., Heslin, D., French, J., Mount, H. T., Nixon, R. A., Mercken, M., Bergeron, C., Fraser, P. E., St George-Hyslop, P. and Westaway, D. (2000). "A beta peptide immunization reduces behavioural impairment and plaques in a model of Alzheimer's disease." Nature **408** (6815): 979-982.

Johnson, N., Ng, T. T. and Parkin, J. M. (1997). "Camptothecin causes cell cycle perturbations within T-lymphoblastoid cells followed by dose dependent induction of apoptosis." Leuk Res **21**(10): 961-972.

Jones, J. and Morgan, B. P. (1995). "Apoptosis is associated with reduced expression of complement regulatory molecules, adhesion molecules and other receptors on polymorphonuclear leucocytes: functional relevance and role in inflammation." Immunology **86**(4): 651-660.

Jourquin, J., Tremblay, E., Decanis, N., Charton, G., Hanessian, S., Chollet, A. M., Le Diguardher, T., Khrestchatisky, M. and Rivera, S. (2003). "Neuronal activity-dependent increase of net matrix metalloproteinase activity is associated with MMP-9 neurotoxicity after kainate." Eur J Neurosci **18**(6): 1507-1517.

Jurianz, K., Ziegler, S., Donin, N., Reiter, Y., Fishelson, Z. and Kirschfink, M. (2001). "K562 erythroleukemic cells are equipped with multiple mechanisms of resistance to lysis by complement." Int J Cancer **93**(6): 848-854.

Jurianz, K., Ziegler, S., Garcia-Schuler, H., Kraus, S., Bohana-Kashtan, O., Fishelson, Z. and Kirschfink, M. (1999). "Complement resistance of tumor cells: basal and induced mechanisms." Mol Immunol **36**(13-14): 929-939.

Kerr, J. F., Wyllie, A. H. and Currie, A. R. (1972). "Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics." Br J Cancer **26**(4): 239-257.

Kim, S. H., Carney, D. F., Hammer, C. H. and Shin, M. L. (1987). "Nucleated cell killing by complement: effects of C5b-9 channel size and extracellular Ca2+ on the lytic process." J Immunol **138**(5): 1530-1536.

Kim, Y. S., Kim, S. S., Cho, J. J., Choi, D. H., Hwang, O., Shin, D. H., Chun, H. S., Beal, M. F. and Joh, T. H. (2005). "Matrix metalloproteinase-3: a novel signaling proteinase from apoptotic neuronal cells that activates microglia." J Neurosci **25**(14): 3701-3711.

Kirschfink, M. (2001). "Targeting complement in therapy." Immunol Rev **180**: 177-189.

Koch, C. A., Kanazawa, A., Nishitai, R., Knudsen, B. E., Ogata, K., Plummer, T. B., Butters, K. and Platt, J. L. (2005). "Intrinsic resistance of hepatocytes to complement-mediated injury." J Immunol **174**(11): 7302-7309.

Korb, L. C. and Ahearn, J. M. (1997). "C1q binds directly and specifically to surface blebs of apoptotic human keratinocytes: complement deficiency and systemic lupus erythematosus revisited." J Immunol **158**(10): 4525-4528.

Kornek, B. and Lassmann, H. (2003). "Neuropathology of multiple sclerosis-new concepts." Brain Res Bull **61**(3): 321-326.

Koski, C. L., Ramm, L. E., Hammer, C. H., Mayer, M. M. and Shin, M. L. (1983). "Cytolysis of nucleated cells by complement: cell death displays multi-hit characteristics." Proc Natl Acad Sci U S A **80**(12): 3816-3820.

Kothakota, S., Azuma, T., Reinhard, C., Klippel, A., Tang, J., Chu, K., McGarry, T. J., Kirschner, M. W., Koths, K., Kwiatkowski, D. J. and Williams, L. T. (1997). "Caspase-3-generated fragment of gelsolin: effector of morphological change in apoptosis." Science **278**(5336): 294-298.

Kraus, S. and Fishelson, Z. (2000). "Cell desensitization by sublytic C5b-9 complexes and calcium ionophores depends on activation of protein kinase C." Eur J Immunol **30**(5): 1272-1280.

Kraus, S., Seger, R. and Fishelson, Z. (2001). "Involvement of the ERK mitogen-activated protein kinase in cell resistance to complement-mediated lysis." Clin Exp Immunol **123**(3): 366-374.

Kurita-Taniguchi, M., Fukui, A., Hazeki, K., Hirano, A., Tsuji, S., Matsumoto, M., Watanabe, M., Ueda, S. and Seya, T. (2000). "Functional modulation of human macrophages through CD46 (measles virus receptor): production of IL-12 p40 and nitric oxide in association with recruitment of protein-tyrosine phosphatase SHP-1 to CD46." J Immunol **165**(9): 5143-5152.

Laemmli, U. K. (1970). "Cleavage of structural proteins during the assembly of the head of bacteriophage T4." Nature **227**(5259): 680-685.

Lee, A., Whyte, M. K. and Haslett, C. (1993). "Inhibition of apoptosis and prolongation of neutrophil functional longevity by inflammatory mediators." J Leukoc Biol **54**(4): 283-288.

Leigh, L. E., Ghebrehiwet, B., Perera, T. P., Bird, I. N., Strong, P., Kishore, U., Reid, K. B. and Eggleton, P. (1998). "C1q-mediated chemotaxis by human neutrophils: involvement of gClqR

and G-protein signalling mechanisms." Biochem J **330 (Pt 1)**: 247-254.

Li, M. O., Sarkisian, M. R., Mehal, W. Z., Rakic, P. and Flavell, R. A. (2003). "Phosphatidylserine receptor is required for clearance of apoptotic cells." Science **302**(5650): 1560-1563.

Linington, C., Morgan, B. P., Scolding, N. J., Wilkins, P., Piddlesden, S. and Compston, D. A. (1989). "The role of complement in the pathogenesis of experimental allergic encephalomyelitis." Brain **112 (Pt 4)**: 895-911.

Linton, S. M. and Morgan, B. P. (1999). "Complement activation and inhibition in experimental models of arthritis." Mol Immunol **36**(13-14): 905-914.

Longhi, M. P., Sivasankar, B., Omidvar, N., Morgan, B. P. and Gallimore, A. (2005). "Cutting Edge: Murine CD59a Modulates Antiviral CD4+ T Cell Activity in a Complement-Independent Manner." J Immunol **175**(11): 7098-7102.

Lothman, E. W. and Collins, R. C. (1981). "Kainic acid induced limbic seizures: metabolic, behavioral, electroencephalographic and neuropathological correlates." Brain Res **218**(1-2): 299-318.

Lue, L. F., Brachova, L., Civin, W. H. and Rogers, J. (1996). "Inflammation, A beta deposition, and neurofibrillary tangle formation as correlates of Alzheimer's disease neurodegeneration." J Neuropathol Exp Neurol **55**(10): 1083-1088.

Marth, T. and Kelsall, B. L. (1997). "Regulation of interleukin-12 by complement receptor 3 signaling." J Exp Med **185**(11): 1987-1995.

Mead, R. J., Neal, J. W., Griffiths, M. R., Linington, C., Botto, M., Lassmann, H. and Morgan, B. P. (2004). "Deficiency of the complement regulator CD59a enhances disease severity, demyelination and axonal injury in murine acute experimental allergic encephalomyelitis." Lab Invest **84**(1): 21-28.

Mead, R. J., Singhrao, S. K., Neal, J. W., Lassmann, H. and Morgan, B. P. (2002). "The membrane attack complex of complement causes severe demyelination associated with acute axonal

injury." J Immunol **168**(1): 458-465.

Meri, S., Morgan, B. P., Davies, A., Daniels, R. H., Olavesen, M. G., Waldmann, H. and Lachmann, P. J. (1990). "Human protectin (CD59), an 18,000-20,000 MW complement lysis restricting factor, inhibits C5b-8 catalysed insertion of C9 into lipid bilayers." Immunology **71**(1): 1-9.

Mevorach, D., Mascarenhas, J. O., Gershov, D. and Elkon, K. B. (1998). "Complement-dependent clearance of apoptotic cells by human macrophages." J Exp Med **188**(12): 2313-2320.

Meyer, R., Weissert, R., Diem, R., Storch, M. K., de Graaf, K. L., Kramer, B. and Bahr, M. (2001). "Acute neuronal apoptosis in a rat model of multiple sclerosis." J Neurosci **21**(16): 6214-6220.

Mishizen-Eberz, A. J., Rissman, R. A., Carter, T. L., Ikonomovic, M. D., Wolfe, B. B. and Armstrong, D. M. (2004). "Biochemical and molecular studies of NMDA receptor subunits NR1/2A/2B in hippocampal subregions throughout progression of Alzheimer's disease pathology." Neurobiol Dis **15**(1): 80-92.

Mizuno, M., Harris, C. L., Johnson, P. M. and Morgan, B. P. (2004). "Rat membrane cofactor protein (MCP; CD46) is expressed only in the acrosome of developing and mature spermatozoa and mediates binding to immobilized activated C3." Biol Reprod **71**(4): 1374-1383.

Mohan, C., Adams, S., Stanik, V. and Datta, S. K. (1993). "Nucleosome: a major immunogen for pathogenic autoantibody-inducing T cells of lupus." J Exp Med **177**(5): 1367-1381.

Monsinjon, T., Richard, V. and Fontaine, M. (2001). "Complement and its implications in cardiac ischemia/reperfusion: strategies to inhibit complement." Fundam Clin Pharmacol **15**(5): 293-306.

Morgan, B. P. (1989). "Complement membrane attack on nucleated cells: resistance, recovery and non-lethal effects." Biochem J **264**(1): 1-14.

Morgan, B. P. (1990). Complement: Clinical Aspects and Relevance to Disease. London, Academic Press.

Morgan, B. P. and Campbell, A. K. (1985). "The recovery of human polymorphonuclear leucocytes from sublytic complement attack is mediated by changes in intracellular free calcium." Biochem J **231**(1): 205-208.

Morgan, B. P., Dankert, J. R. and Esser, A. F. (1987). "Recovery of human neutrophils from complement attack: removal of the membrane attack complex by endocytosis and exocytosis." J Immunol **138**(1): 246-253.

Morgan, B. P. and Harris, C. L. (1999). Complement Regulatory Proteins. London, Academic Press.

Morgan, B. P., Luzio, J. P. and Campbell, A. K. (1986). "Intracellular Ca2+ and cell injury: a paradoxical role of Ca2+ in complement membrane attack." Cell Calcium **7**(5-6): 399-411.

Morgan, B. P. and Walport, M. J. (1991). "Complement deficiency and disease." Immunol Today **12** (9): 301-306.

Morgan, D., Diamond, D. M., Gottschall, P. E., Ugen, K. E., Dickey, C., Hardy, J., Duff, K., Jantzen, P., DiCarlo, G., Wilcock, D., Connor, K., Hatcher, J., Hope, C., Gordon, M. and Arendash, G. W. (2000). "A beta peptide vaccination prevents memory loss in an animal model of Alzheimer's disease." Nature **408**(6815): 982-985.

Moroni, G., Trendelenburg, M., Del Papa, N., Quaglini, S., Raschi, E., Panzeri, P., Testoni, C., Tincani, A., Banfi, G., Balestrieri, G., Schifferli, J. A., Meroni, P. L. and Ponticelli, C. (2001). "Anti-C1q antibodies may help in diagnosing a renal flare in lupus nephritis." Am J Kidney Dis **37**(3): 490-498.

Morris, E. J. and Geller, H. M. (1996). "Induction of neuronal apoptosis by camptothecin, an inhibitor of DNA topoisomerase-I: evidence for cell cycle-independent toxicity." J Cell Biol **134**(3): 757-770.

Mukherjee, P. and Pasinetti, G. M. (2001). "Complement anaphylatoxin C5a neuroprotects through mitogen-activated protein kinase-dependent inhibition of caspase 3." J Neurochem **77**(1): 43-

49.

Napirei, M., Karsunky, H., Zevnik, B., Stephan, H., Mannherz, H. G. and Moroy, T. (2000). "Features of systemic lupus erythematosus in Dnase1-deficient mice." Nat Genet **25**(2): 177-181.

Nauta, A. J., Daha, M. R., Tijsma, O., van de Water, B., Tedesco, F. and Roos, A. (2002). "The membrane attack complex of complement induces caspase activation and apoptosis." Eur J Immunol **32**(3): 783-792.

Nauta, A. J., Raaschou-Jensen, N., Garred, P., Daha, M. R., Madsen, H. O., Ryder, L. P., Koch, C., Borrias, M. and Roos, A. (2002). "Mannose-Binding Lectin (MBL) Tags Apoptotic Cells and Cell Blebs as well as Necrotic Cells." International Pharmacology **2**(9): 1348.

Nauta, A. J., Raaschou-Jensen, N., Roos, A., Daha, M. R., Madsen, H. O., Borrias-Essers, M. C., Ryder, L. P., Koch, C. and Garred, P. (2003). "Mannose-binding lectin engagement with late apoptotic and necrotic cells." Eur J Immunol **33**(10): 2853-2863.

Niculescu, F., Badea, T. and Rus, H. (1999). "Sublytic C5b-9 induces proliferation of human aortic smooth muscle cells: role of mitogen activated protein kinase and phosphatidylinositol 3-kinase." Atherosclerosis **142**(1): 47-56.

Niculescu, F. and Rus, H. (1999). "Complement activation and atherosclerosis." Mol Immunol **36**(13-14): 949-955.

Niculescu, T., Weerth, S., Niculescu, F., Cudrici, C., Rus, V., Raine, C. S., Shin, M. L. and Rus, H. (2004). "Effects of complement C5 on apoptosis in experimental autoimmune encephalomyelitis." J Immunol **172**(9): 5702-5706.

Ogden, C. A., deCathelineau, A., Hoffmann, P. R., Bratton, D., Ghebrehiwet, B., Fadok, V. A. and Henson, P. M. (2001). "C1q and mannose binding lectin engagement of cell surface calreticulin and CD91 initiates macropinocytosis and uptake of apoptotic cells." J Exp Med **194**(6): 781-795.

Osaka, H., McGinty, A., Hoepken, U. E., Lu, B., Gerard, C. and Pasinetti, G. M. (1999). "Expression of C5a receptor in mouse brain: role in signal transduction and neurodegeneration." Neuroscience **88**(4): 1073-1082.

Osaka, H., Mukherjee, P., Aisen, P. S. and Pasinetti, G. M. (1999). "Complement-derived anaphylatoxin C5a protects against glutamate-mediated neurotoxicity." J Cell Biochem **73**(3): 303-311.

Papadimitriou, J. C., Phelps, P. C., Shin, M. L., Smith, M. W. and Trump, B. F. (1994). "Effects of Ca2+ deregulation on mitochondrial membrane potential and cell viability in nucleated cells following lytic complement attack." Cell Calcium **15**(3): 217-227.

Papadimitriou, J. C., Ramm, L. E., Drachenberg, C. B., Trump, B. F. and Shin, M. L. (1991). "Quantitative analysis of adenine nucleotides during the prelytic phase of cell death mediated by C5b-9." J Immunol **147**(1): 212-217.

Parone, P., Priault, M., James, D., Nothwehr, S. F. and Martinou, J. C. (2003). "Apoptosis: bombarding the mitochondria." Essays Biochem **39**: 41-51.

Pasinetti, G. M., Tocco, G., Sakhi, S., Musleh, W. D., DeSimoni, M. G., Mascarucci, P., Schreiber, S., Baudry, M. and Finch, C. E. (1996). "Hereditary deficiencies in complement C5 are associated with intensified neurodegenerative responses that implicate new roles for the C-system in neuronal and astrocytic functions." Neurobiol Dis **3**(3): 197-204.

Peng, H., Takano, T., Papillon, J., Bijian, K., Khadir, A. and Cybulsky, A. V. (2002). "Complement activates the c-Jun N-terminal kinase/stress-activated protein kinase in glomerular epithelial cells." J Immunol **169**(5): 2594-2601.

Penkowa, M., Florit, S., Giralt, M., Quintana, A., Molinero, A., Carrasco, J. and Hidalgo, J. (2005). "Metallothionein reduces central nervous system inflammation, neurodegeneration, and cell death following kainic acid-induced epileptic seizures." J Neurosci Res **79**(4): 522-534.

Peterson, J. W., Bo, L., Mork, S., Chang, A. and Trapp, B. D. (2001). "Transected neurites, apoptotic neurons, and reduced inflammation in cortical multiple sclerosis lesions." Ann Neurol **50**(3): 389-400.

Piddlesden, S. J., Storch, M. K., Hibbs, M., Freeman, A. M., Lassmann, H. and Morgan, B. P. (1994). "Soluble recombinant complement receptor 1 inhibits inflammation and demyelination in antibody-mediated demyelinating experimental allergic encephalomyelitis." J Immunol **152** (11): 5477-5484.

Pitt, D., Werner, P. and Raine, C. S. (2000). "Glutamate excitotoxicity in a model of multiple sclerosis." Nat Med **6**(1): 67-70.

Pittock, S. J., McClelland, R. L., Achenbach, S. J., Konig, F., Bitsch, A., Bruck, W., Lassmann, H., Parisi, J. E., Scheithauer, B. W., Rodriguez, M., Weinshenker, B. G. and Lucchinetti, C. F. (2005). "Clinical course, pathological correlations, and outcome of biopsy proved inflammatory demyelinating disease." J Neurol Neurosurg Psychiatry **76**(12): 1693-1697.

Pleasure, S. J., Page, C. and Lee, V. M. (1992). "Pure, postmitotic, polarized human neurons derived from NTera 2 cells provide a system for expressing exogenous proteins in terminally differentiated neurons." J Neurosci **12**(5): 1802-1815.

Podack, E. R. and Tschopp, J. (1984). "Membrane attack by complement." Mol Immunol **21**(7): 589-603.

Pollard, H., Charriaut-Marlangue, C., Cantagrel, S., Represa, A., Robain, O., Moreau, J. and Ben-Ari, Y. (1994). "Kainate-induced apoptotic cell death in hippocampal neurons." Neuroscience **63** (1): 7-18.

Porcel, J. M., Peakman, M., Senaldi, G. and Vergani, D. (1993). "Methods for assessing complement activation in the clinical immunology laboratory." J Immunol Methods **157**(1-2): 1-9.

Prineas, J. W., Kwon, E. E., Cho, E. S., Sharer, L. R., Barnett, M. H., Oleszak, E. L., Hoffman, B. and Morgan, B. P. (2001). "Immunopathology of secondary-progressive multiple sclerosis."

Ann Neurol **50**(5): 646-657.

Puig, B. and Ferrer, I. (2002). "Caspase-3-associated apoptotic cell death in excitotoxic necrosis of the entorhinal cortex following intraperitoneal injection of kainic acid in the rat." Neurosci Lett **321**(3): 182-186.

Putzu, G. A., Figarella-Branger, D., Bouvier-Labit, C., Liprandi, A., Bianco, N. and Pellissier, J. F. (2000). "Immunohistochemical localization of cytokines, C5b-9 and ICAM-1 in peripheral nerve of Guillain-Barre syndrome." J Neurol Sci **174**(1): 16-21.

Rao, L., Perez, D. and White, E. (1996). "Lamin proteolysis facilitates nuclear events during apoptosis." J Cell Biol **135**(6 Pt 1): 1441-1455.

Reiter, Y., Ciobotariu, A. and Fishelson, Z. (1992). "Sublytic complement attack protects tumor cells from lytic doses of antibody and complement." Eur J Immunol **22**(5): 1207-1213.

Reiter, Y., Ciobotariu, A., Jones, J., Morgan, B. P. and Fishelson, Z. (1995). "Complement membrane attack complex, perforin, and bacterial exotoxins induce in K562 cells calcium-dependent cross-protection from lysis." J Immunol **155**(4): 2203-2210.

Reiter, Y. and Fishelson, Z. (1992). "Complement membrane attack complexes induce in human leukemic cells rapid expression of large proteins (L-CIP)." Mol Immunol **29**(6): 771-781.

Riedemann, N. C., Guo, R. F., Laudes, I. J., Keller, K., Sarma, V. J., Padgaonkar, V., Zetoune, F. S. and Ward, P. A. (2002). "C5a receptor and thymocyte apoptosis in sepsis." Faseb J **16**(8): 887-888.

Robertson, G. S., Crocker, S. J., Nicholson, D. W. and Schulz, J. B. (2000). "Neuroprotection by the inhibition of apoptosis." Brain Pathol **10**(2): 283-292.

Roederer, M. (2004). Conjugation of monoclonal antibodies, http://www.drmr.com/abcon/.

Rosenberg, G. A. (2002). "Matrix metalloproteinases in neuroinflammation." Glia **39**(3): 279-291.

Rosenberg, G. A. (2005). "Matrix metalloproteinases biomarkers in multiple sclerosis." Lancet **365** (9467): 1291-1293.

Rosenblum, M. D., Olasz, E., Woodliff, J. E., Johnson, B. D., Konkol, M. C., Gerber, K. A., Orentas, R. J., Sandford, G. and Truitt, R. L. (2004). "CD200 is a novel p53-target gene involved in apoptosis-associated immune tolerance." Blood **103**(7): 2691-2698.

Rozovsky, I., Morgan, T. E., Willoughby, D. A., Dugichi-Djordjevich, M. M., Pasinetti, G. M., Johnson, S. A. and Finch, C. E. (1994). "Selective expression of clusterin (SGP-2) and complement C1qB and C4 during responses to neurotoxins in vivo and in vitro." Neuroscience **62**(3): 741-758.

Rus, H., Niculescu, F., Badea, T. and Shin, M. L. (1997). "Terminal complement complexes induce cell cycle entry in oligodendrocytes through mitogen activated protein kinase pathway." Immunopharmacology **38**(1-2): 177-187.

Rus, H., Weerth, S., Sloane, L., Niculescu, F., Rus, V., Raine, C. and Shin, M. L. (2002). "The effect of terminal complement complex on apoptosis gene expression in experimental autoimmune encephalomyelitis." Int Pharmacol **2**(9): 1347.

Rus, H. G., Niculescu, F. and Shin, M. L. (1996). "Sublytic complement attack induces cell cycle in oligodendrocytes." J Immunol **156**(12): 4892-4900.

Rus, H. G., Niculescu, F. I. and Shin, M. L. (2001). "Role of the C5b-9 complement complex in cell cycle and apoptosis." Immunol Rev **180**: 49-55.

Sala-Newby, G. B., Taylor, K. M., Badminton, M. N., Rembold, C. M. and Campbell, A. K. (1998). "Imaging bioluminescent indicators shows Ca2+ and ATP permeability thresholds in live cells attacked by complement." Immunology **93**(4): 601-609.

Sanchez, A., Feito, M. J. and Rojo, J. M. (2004). "CD46-mediated costimulation induces a Th1-biased response and enhances early TCR/CD3 signaling in human CD4+ T lymphocytes." Eur J Immunol **34**(9): 2439-2448.

Sato, T., Van Dixhoorn, M. G., Prins, F. A., Mooney, A., Verhagen, N., Muizert, Y., Savill, J., Van

Es, L. A. and Daha, M. R. (1999). "The terminal sequence of complement plays an essential role in antibody-mediated renal cell apoptosis." J Am Soc Nephrol **10**(6): 1242-1252.

Savill, J. and Fadok, V. (2000). "Corpse clearance defines the meaning of cell death." Nature **407** (6805): 784-788.

Savill, J. S., Wyllie, A. H., Henson, J. E., Walport, M. J., Henson, P. M. and Haslett, C. (1989). "Macrophage phagocytosis of aging neutrophils in inflammation. Programmed cell death in the neutrophil leads to its recognition by macrophages." J Clin Invest **83**(3): 865-875.

Schwab, B. L., Guerini, D., Didszun, C., Bano, D., Ferrando-May, E., Fava, E., Tam, J., Xu, D., Xanthoudakis, S., Nicholson, D. W., Carafoli, E. and Nicotera, P. (2002). "Cleavage of plasma membrane calcium pumps by caspases: a link between apoptosis and necrosis." Cell Death Differ **9**(8): 818-831.

Scolding, N. J., Morgan, B. P. and Compston, D. A. (1998). "The expression of complement regulatory proteins by adult human oligodendrocytes." J Neuroimmunol **84**(1): 69-75.

Scolding, N. J., Morgan, B. P., Houston, A., Campbell, A. K., Linington, C. and Compston, D. A. (1989). "Normal rat serum cytotoxicity against syngeneic oligodendrocytes. Complement activation and attack in the absence of anti-myelin antibodies." J Neurol Sci **89**(2-3): 289-300.

Scolding, N. J., Morgan, B. P., Houston, W. A., Linington, C., Campbell, A. K. and Compston, D. A. (1989). "Vesicular removal by oligodendrocytes of membrane attack complexes formed by activated complement." Nature **339**(6226): 620-622.

Shen, Y., Sullivan, T., Lee, C. M., Meri, S., Shiosaki, K. and Lin, C. W. (1998). "Induced expression of neuronal membrane attack complex and cell death by Alzheimer's beta-amyloid peptide." Brain Res **796**(1-2): 187-197.

Shimizu, A., Masuda, Y., Kitamura, H., Ishizaki, M., Ohashi, R., Sugisaki, Y. and Yamanaka, N. (2000). "Complement-mediated killing of mesangial cells in experimental glomerulonephritis: cell death by a combination of apoptosis and necrosis." Nephron **86**(2): 152-160.

Simak, J., Holada, K. and Vostal, J. G. (2002). "Release of annexin V-binding membrane microparticles from cultured human umbilical vein endothelial cells after treatment with camptothecin." BMC Cell Biol **3**(1): 11.

Singhrao, S. K., Neal, J. W., Rushmere, N. K., Morgan, B. P. and Gasque, P. (1999). "Differential expression of individual complement regulators in the brain and choroid plexus." Lab Invest **79**(10): 1247-1259.

Singhrao, S. K., Neal, J. W., Rushmere, N. K., Morgan, B. P. and Gasque, P. (2000). "Spontaneous classical pathway activation and deficiency of membrane regulators render human neurons susceptible to complement lysis." Am J Pathol **157**(3): 905-918.

Soane, L., Cho, H. J., Niculescu, F., Rus, H. and Shin, M. L. (2001). "C5b-9 terminal complement complex protects oligodendrocytes from death by regulating Bad through phosphatidylinositol 3-kinase/Akt pathway." J Immunol **167**(4): 2305-2311.

Soane, L., Rus, H., Niculescu, F. and Shin, M. L. (1999). "Inhibition of oligodendrocyte apoptosis by sublytic C5b-9 is associated with enhanced synthesis of bcl-2 and mediated by inhibition of caspase-3 activation." J Immunol **163**(11): 6132-6138.

Sperk, G., Lassmann, H., Baran, H., Seitelberger, F. and Hornykiewicz, O. (1985). "Kainic acid-induced seizures: dose-relationship of behavioural, neurochemical and histopathological changes." Brain Res **338**(2): 289-295.

Spiller, O. B., Moretto, G., Kim, S. U., Morgan, B. P. and Devine, D. V. (1996). "Complement expression on astrocytes and astrocytoma cell lines: failure of complement regulation at the C3 level correlates with very low CD55 expression." J Neuroimmunol **71**(1-2): 97-106.

Stolzing, A. and Grune, T. (2004). "Neuronal apoptotic bodies: phagocytosis and degradation by primary microglial cells." Faseb J **18**(6): 743-745.

Storch, M. K., Piddlesden, S., Haltia, M., Iivanainen, M., Morgan, P. and Lassmann, H. (1998).

"Multiple sclerosis: in situ evidence for antibody- and complement-mediated demyelination." Ann Neurol **43**(4): 465-471.

Takano, T., Cybulsky, A. V., Yang, X. and Aoudjit, L. (2001). "Complement C5b-9 induces cyclooxygenase-2 gene transcription in glomerular epithelial cells." Am J Physiol Renal Physiol **281**(5): F841-850.

Takizawa, F., Tsuji, S. and Nagasawa, S. (1996). "Enhancement of macrophage phagocytosis upon iC3b deposition on apoptotic cells." FEBS Lett **397**(2-3): 269-272.

Taylor, P. R., Carugati, A., Fadok, V. A., Cook, H. T., Andrews, M., Carroll, M. C., Savill, J. S., Henson, P. M., Botto, M. and Walport, M. J. (2000). "A hierarchical role for classical pathway complement proteins in the clearance of apoptotic cells in vivo." J Exp Med **192**(3): 359-366.

Thomas, A., Gasque, P., Vaudry, D., Gonzalez, B. and Fontaine, M. (2000). "Expression of a complete and functional complement system by human neuronal cells in vitro." Int Immunol **12**(7): 1015-1023.

Tilley, S. J., Orlova, E. V., Gilbert, R. J., Andrew, P. W. and Saibil, H. R. (2005). "Structural basis of pore formation by the bacterial toxin pneumolysin." Cell **121**(2): 247-256.

Trapp, B. D., Peterson, J., Ransohoff, R. M., Rudick, R., Mork, S. and Bo, L. (1998). "Axonal transection in the lesions of multiple sclerosis." N Engl J Med **338**(5): 278-285.

Tsuji, S., Kaji, K. and Nagasawa, S. (1994). "Activation of the alternative pathway of human complement by apoptotic human umbilical vein endothelial cells." J Biochem (Tokyo) **116**(4): 794-800.

Tsunoda, S., Kawano, M., Koni, I., Kasahara, Y., Yachie, A., Miyawaki, T. and Seki, H. (2000). "Diminished expression of CD59 on activated CD8+ T cells undergoing apoptosis in systemic lupus erythematosus and Sjogren's syndrome." Scand J Immunol **51**(3): 293-299.

Vakeva, A. P., Agah, A., Rollins, S. A., Matis, L. A., Li, L. and Stahl, G. L. (1998). "Myocardial

infarction and apoptosis after myocardial ischemia and reperfusion: role of the terminal complement components and inhibition by anti-C5 therapy." Circulation **97**(22): 2259-2267.

Van Beek, J., Bernaudin, M., Petit, E., Gasque, P., Nouvelot, A., MacKenzie, E. T. and Fontaine, M. (2000). "Expression of receptors for complement anaphylatoxins C3a and C5a following permanent focal cerebral ischemia in the mouse." Exp Neurol **161**(1): 373-382.

van Beek, J., Nicole, O., Ali, C., Ischenko, A., MacKenzie, E. T., Buisson, A. and Fontaine, M. (2001). "Complement anaphylatoxin C3a is selectively protective against NMDA-induced neuronal cell death." Neuroreport **12**(2): 289-293.

van Beek, J., van Meurs, M., t Hart, B. A., Brok, H. P., Neal, J. W., Chatagner, A., Harris, C. L., Omidvar, N., Morgan, B. P., Laman, J. D. and Gasque, P. (2005). "Decay-accelerating factor (CD55) is expressed by neurons in response to chronic but not acute autoimmune central nervous system inflammation associated with complement activation." J Immunol **174**(4): 2353-2365.

Van Den Berg, C. W., De Andrade, R. M., Magnoli, F. C., Marchbank, K. J. and Tambourgi, D. V. (2002). "Loxosceles spider venom induces metalloproteinase mediated cleavage of MCP/CD46 and MHCI and induces protection against C-mediated lysis." Immunology **107**(1): 102-110.

Vogt, W., Zimmermann, B., Hesse, D. and Nolte, R. (1992). "Activation of the fifth component of human complement, C5, without cleavage, by methionine oxidizing agents." Mol Immunol **29** (2): 251-256.

Wajant, H. (2003). "Death receptors." Essays Biochem **39**: 53-71.

Walport, M. J. (2001). "Complement. First of two parts." N Engl J Med **344**(14): 1058-1066.

Walport, M. J. (2001). "Complement. Second of two parts." N Engl J Med **344**(15): 1140-1144.

Weerth, S. H., Rus, H., Shin, M. L. and Raine, C. S. (2003). "Complement C5 in experimental autoimmune encephalomyelitis (EAE) facilitates remyelination and prevents gliosis." Am J Pathol **163**(3): 1069-1080.

Welch, T. R. (2002). "Complement in glomerulonephritis." Nat Genet **31**(4): 333-334.

Wood, A., Wing, M. G., Benham, C. D. and Compston, D. A. (1993). "Specific induction of intracellular calcium oscillations by complement membrane attack on oligodendroglia." J Neurosci **13**(8): 3319-3332.

Wright, G. J., Jones, M., Puklavec, M. J., Brown, M. H. and Barclay, A. N. (2001). "The unusual distribution of the neuronal/lymphoid cell surface CD200 (OX2) glycoprotein is conserved in humans." Immunology **102**(2): 173-179.

Wyllie, A. H. (1980). "Glucocorticoid-induced thymocyte apoptosis is associated with endogenous endonuclease activation." Nature **284**(5756): 555-556.

Wyss-Coray, T., Yan, F., Lin, A. H., Lambris, J. D., Alexander, J. J., Quigg, R. J. and Masliah, E. (2002). "Prominent neurodegeneration and increased plaque formation in complement-inhibited Alzheimer's mice." Proc Natl Acad Sci U S A **99**(16): 10837-10842.

Xiong, Z. Q. and McNamara, J. O. (2002). "Fleeting activation of ionotropic glutamate receptors sensitizes cortical neurons to complement attack." Neuron **36**(3): 363-374.

Xiong, Z. Q., Qian, W., Suzuki, K. and McNamara, J. O. (2003). "Formation of complement membrane attack complex in mammalian cerebral cortex evokes seizures and neurodegeneration." J Neurosci **23**(3): 955-960.

Yakovlev, A. G. and Faden, A. I. (2001). "Caspase-dependent apoptotic pathways in CNS injury." Mol Neurobiol **24**(1-3): 131-144.

Yang, L. B., Li, R., Meri, S., Rogers, J. and Shen, Y. (2000). "Deficiency of complement defense protein CD59 may contribute to neurodegeneration in Alzheimer's disease." J Neurosci **20** (20): 7505-7509.

Yoshioka, A., Ikegaki, N., Williams, M. and Pleasure, D. (1996). "Expression of N-methyl-D-aspartate (NMDA) and non-NMDA glutamate receptor genes in neuroblastoma, medulloblastoma, and other cells lines." J Neurosci Res **46**(2): 164-178.

Zajicek, J., Wing, M., Skepper, J. and Compston, A. (1995). "Human oligodendrocytes are not sensitive to complement. A study of CD59 expression in the human central nervous system." Lab Invest **73**(1): 128-138.

Zwaka, T. P., Manolov, D., Ozdemir, C., Marx, N., Kaya, Z., Kochs, M., Hoher, M., Hombach, V. and Torzewski, J. (2002). "Complement and dilated cardiomyopathy: a role of sublytic terminal complement complex-induced tumor necrosis factor-alpha synthesis in cardiac myocytes." Am J Pathol **161**(2): 449-457.

Zwart, B., Ciurana, C., Rensink, I., Manoe, R., Hack, C. E. and Aarden, L. A. (2004). "Complement activation by apoptotic cells occurs predominantly via IgM and is limited to late apoptotic (secondary necrotic) cells." Autoimmunity **37**(2): 95-102.

Appendices

REVIEW

Beyond lysis: how complement influences cell fate

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ABSTRACT

Complement is a central component of the innate immune system involved in protection against pathogens. For many years, complement has been known to cause death of targets, either indirectly by attracting and activating phagocytes or directly by formation of a membrane pore, the membrane attack complex. More recently, it has been recognized that complement may cause other 'non-classical' effects that may not directly be aimed at killing of pathogens. Products of complement activation collaborate with the adaptive immune system to enhance responses to antigens. The membrane attack complex of complement, apart from lysing cells, can also trigger diverse events in target cells that include cell activation, proliferation, resistance to subsequent complement attack and either resistance to, or induction of, apoptosis. Various complement products play important roles in signalling for clearance by phagocytes of apoptotic self cells. Here we review some of these non-classical activities of complement and stress the roles that they may play in maintaining the integrity of the organism.

INTRODUCTION

Complement is a central part of the innate immune system, on the front line in defence of the body from invading pathogens and in clearance of potentially damaging debris. From its very earliest description over a century ago, complement has been inextricably linked with cell death [1]. The complement system was first identified as a heat-sensitive fraction of serum that acted in concert with antibody to kill bacteria [2]. A century on, research in the field is flourishing, and the fruits of the work are influencing clinical practice, with a number of therapeutics designed to modulate complement activity currently undergoing clinical trials [3].

Of course, complement does more than just kill cells. It has varied and wide-ranging functions that include a crucial role in the efficient phagocytosis of pathogens and cellular debris by opsonizing them with molecules such as C3b [4]. By doing so, it aids the solubilization and clearance of immune complexes that would otherwise lodge in capillary beds and cause damage. Another of its important functions is to act as a stimulus to inflammation, and here the small anaphylatoxic fragments C5a and C3a are involved by directly activating cells bearing the appropriate receptors. More recently, an important role for complement in linking innate and adaptive immunity has been revealed, for example by contributing a second signal to B lymphocytes that have recognized a complement-opsonized antigen [5].

The complement system itself is remarkable in its simplicity. Unfortunately, this simplicity is lost in a quagmire of 'difficult' terminology, a relic of history that has never been adequately addressed. Once over the terminology hurdle, the system reveals its true nature (Figure 1A). It consists of three recognition systems that permit the identification of appropriate targets, several

Key words: apoptosis, C5a, cell death, complement, membrane attack complex, non-lethal effects.

Abbreviations: bFGF, basic fibroblast growth factor; C#, complement component #; CR#, complement receptor #; L-CIP, large complement-induced protein; MAC, membrane attack complex; MAP kinase, mitogen-activated protein kinase; MS, multiple sclerosis; NFκB, nuclear factor κB; PDGF, platelet-derived growth factor; SAP, serum amyloid P; SLE, systemic lupus erythematosus; TNF-α, tumour necrosis factor-α.

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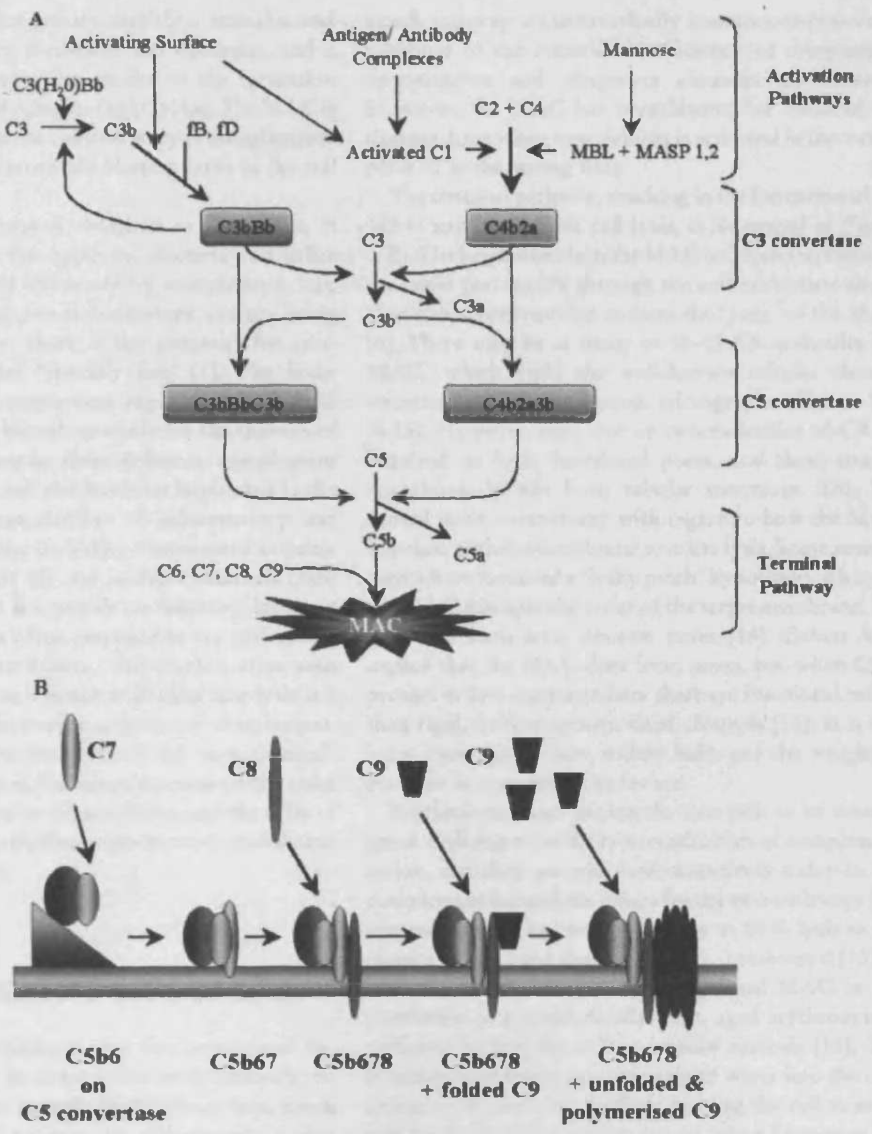

Figure 1 For legend, see facing page

enzyme complexes that greatly amplify a stimulus and produce inflammatory mediators and opsonins, and a single terminal pathway that results in the formation of the membrane attack complex (MAC) [4,6]. The MAC is the main perpetrator in the classical story of complement-mediated cell death, known for blasting holes in the cell membrane.

Although complement is designed to protect us, it sometimes does just the opposite. Bacteria and other organisms are targeted efficiently by complement, but, with such a powerful pro-inflammatory system being activated in the body, there is the potential for considerable damage from 'friendly fire' [1]. The body therefore has many complement regulators, both fluid phase and membrane bound, to minimize the chances of this occurring [7]. Despite these defences, complement still causes damage to self and has been implicated in the pathogenesis of a large number of inflammatory and immunological diseases, including rheumatoid arthritis [8], glomerulonephritis [9] and multiple sclerosis (MS) [10,11]. Although not necessarily an initiating factor in these conditions, it is often responsible for promoting and perpetuating inflammation. Cell death is often seen in these conditions, but it is not at all clear that lysis is a dominant feature in the *in vivo* activities of complement.

This review will describe some of the 'non-classical' activities of complement, focusing attention on the roles of the MAC as a trigger to cell activation, and the roles of the MAC and other complement products as modulators of apoptotic cell death.

COMPLEMENT-MEDIATED LYSIS OF CELLS

As noted above, complement was first recognized because of its capacity, in conjunction with antibody, to lyse cells, in particular bacteria [2,12]. Since then, much attention has focused on how the complement system achieves this, notably the dissection of the molecular mechanisms leading to assembly of the MAC and subsequent cell death. Lysis is an important part of the response to infectious organisms, utilized as a defence not only in the early stages of infection, but also once adaptive immunity has developed [1,5]. Despite this, individuals deficient in components of the membrane attack pathway are not markedly immunocompromised, a tribute to the remarkable efficiency of complement opsonization and phagocyte clearance mechanisms. However, the MAC has been blamed for much of the damage done when complement is activated in the wrong place or at the wrong time.

The terminal pathway, resulting in the formation of the MAC and subsequent cell lysis, is illustrated in Figure 1(B). The key molecule in the MAC is C9, an amphipathic molecule that inserts through the cell membrane and is then able to polymerize to form the 'pore' of the MAC [6]. There may be as many as 16–18 C9 molecules per MAC, which yield the well-known tubular channel structure visible in electron micrographs (Figure 1C) [6,13]. However, only one or two molecules of C9 are required to form functional pores, and these smaller complexes do not form tubular structures. This has caused some controversy with regard to how the MAC interacts with the membrane to cause lysis. Some investigators have favoured a 'leaky patch' hypothesis, whereby the MAC disrupts the order of the target membrane, but does not form true, discrete pores [14]. Others have argued that the MAC does form pores, but when C9 is present in low copy numbers these are functional rather than rigid, hollow protein-lined channels [13]. It is this latter view that is now widely held, and the weight of evidence is certainly in its favour.

Erythrocytes were among the first cells to be investigated with regard to the lytic mechanism of complement action, and they are still used extensively today in the complement haemolytic assays for the two pathways [i.e. measurement of haemolytic activity at 50% lysis in the classical (CH_{50}) and alternative (AH_{50}) pathways] [15]. It has been shown that a single functional MAC in the membrane of a metabolically inert, aged erythrocyte is sufficient to lyse the cell by colloid osmosis [16]. The breached membrane permits entry of water into the cell, driven by the osmotic gradient, causing the cell to swell and burst. A similar picture occurs when liposomes are attacked [17]. Not surprisingly, the efficiency with which lysis occurs depends on the number of MACs in the cell membrane, and on the composition of the extracellular fluid.

This simple picture of osmotic cell lysis does not extend to analyses of MAC-mediated killing of nucleated cells. Metabolically active nucleated cells are, in general,

Figure 1 Complement cascade (A) and formation of the MAC (B, C)

(A) In the complement cascade, activation occurs via the classical, alternative and lectin pathways, which act to target the system. This results in the formation of the C3 and then the C5 convertases, which amplify the response and also release the anaphylatoxins C3a and C5a. The terminal pathway results in the formation of the MAC. Bb, cleaved fragment of factor B; fB, factor B; fD, factor D; MBL, mannan-binding lectin; MASP, MBL-associated serine protease. (B) In MAC formation, C5b binds C6 while still bound to the C5 convertase. On binding C7, the tri-molecular C5b67 is released and inserts into the membrane. C8 then inserts, traversing the membrane. C9 binds initially in its unfolded state, and then unfolds and polymerizes to form the MAC pore. (C) Electron micrograph of the MAC on the surface of a vesicle shed from a neutrophil membrane. The arrows indicate tubular MAC structures seen face on and side on.

much more resistant to the lytic effects of complement when compared with erythrocytes [18]. Complement-mediated lysis of nucleated cells displays 'multi-hit' kinetics, implying a requirement for many MACs on the cell surface, and factors other than colloid osmotic dysregulation, notably the presence of calcium in the extracellular fluid, influence the efficiency of killing [16,19]. In general, nucleated cells differ from aged erythrocytes in that they possess a variety of protective mechanisms that restrict complement-mediated lysis. These include ion pumps that can compensate for membrane pores and the capacity to remove MACs from the cell surface [18,20].

Calcium plays a crucial role in deciding the fate of a cell attacked by complement. The earliest detectable event following MAC attack is a large influx of calcium into the cell [21,22], and increasing levels of extracellular calcium speed the progression to cell death [23]. The ensuing crisis is centred on the mitochondrion. Excess calcium causes loss of the mitochondrial transmembrane potential, resulting in an energy crisis in the cell, as energy-consuming ion pumps frantically try to redress the balance [24]. This precarious situation is exacerbated further by the loss of ATP and its precursors via the MAC pore into the extracellular environment [25]. The decrease in ATP through consumption and leakage renders the cell incapable of sustaining its essential metabolic processes, leading to necrosis.

As indicated above, the MAC is not essential for the killing of most bacteria. Gram-positive bacteria possess an efficient MAC avoidance strategy. The thick cell wall characteristic of these organisms prevents the MAC from reaching and breaching the inner plasma membrane of the bacterium [1]. MAC deposition on the cell wall is without consequence. Gram-negative organisms, however, lack this thick protective coat. Complement activation first permeabilizes the outer membrane, then aids degradation of the thin cell wall and finally exposes the inner membrane to MAC formation and lysis. MAC may also target the zones of apposition of the inner and outer membranes and breach both at the same time [26,27]. Lysis by MAC is a major route for killing of Gram-negative organisms of the genus *Neisseria*; as a consequence, individuals deficient in the components of the MAC, such as C6 and C7, have markedly increased susceptibility to infections by *Neisseria* species that frequently cause meningitis [28].

This section has focused on the 'classical' capacity of MAC to kill cells, and has highlighted the fact that nucleated cells are often very difficult to kill. The recognition of this fact raised the possibility that MAC deposition on nucleated cells might cause other effects relevant to health and disease. These 'non-lethal' consequences of MAC assembly on nucleated cells are increasingly recognized as key events in life-or-death decisions in cells.

CELL SURVIVAL IN THE FACE OF COMPLEMENT MEMBRANE ATTACK

We have already noted that metabolically active nucleated cells are more resistant to complement attack than erythrocytes because of the presence of ion pumps and mechanisms for removal of the MAC [16,18]. Additional protection is provided by the presence of membrane-bound complement regulators [7]. Cells are protected from MAC formation by a membrane-bound molecule, CD59, that blocks assembly of the lytic pore [29]. In addition, nucleated cells may express ecto-proteases on their surface that can cleave complement components, or ecto-kinases that can inactivate them by phosphorylation [30]. Finally, when complement is activated on cells, surviving cells can become protected against subsequent attack [30,31]. The mechanisms of this 'induced protection' phenomenon are uncertain, although it has been shown to depend upon RNA and protein synthesis [31]. A protein complex known as the large complement-induced protein (L-CIP), related to heat-shock proteins, has been shown to be induced, which translocates to the cell membrane, although a protective function of L-CIP has not been shown [32,33]. 'Induced protection' is not limited to MAC attack. Non-lethal amounts of the MAC also protect against other pore formers such as perforin, melittin and streptolysin O which, in turn, can induce protection from MAC attack [34]. All of these protective mechanisms are important in limiting damage to host cells in areas of inflammation, but may also be put to more sinister purpose, for example when they are utilized by tumour cells in order to evade complement-mediated killing.

Non-lytic consequences of complement membrane attack

Removal of MACs represents an important mechanism of cell resistance to, and recovery from, complement attack, and is also one of the best defined of the non-lethal effects of MAC assembly (Figure 2). MACs are removed either by shedding on membrane vesicles (ectocytosis) or by internalization and degradation, depending on the cell type [20,35,36]. The efficiency with which this occurs is temperature dependent [18]. Signalling of MAC removal has been studied and, again, calcium is implicated, acting in its well-known capacity as a second messenger [37]. Calcium influx occurs via the pore, but even in the absence of extracellular calcium the MAC still induces an increase in intracellular calcium by triggering calcium release from stores [38]. Protein kinase C activation occurs, both triggered directly by MAC and signalled by calcium. Events further downstream are poorly defined and the precise mechanism of MAC shedding is completely unknown. Calcium therefore plays a double role, being important in protecting cells when the MAC is

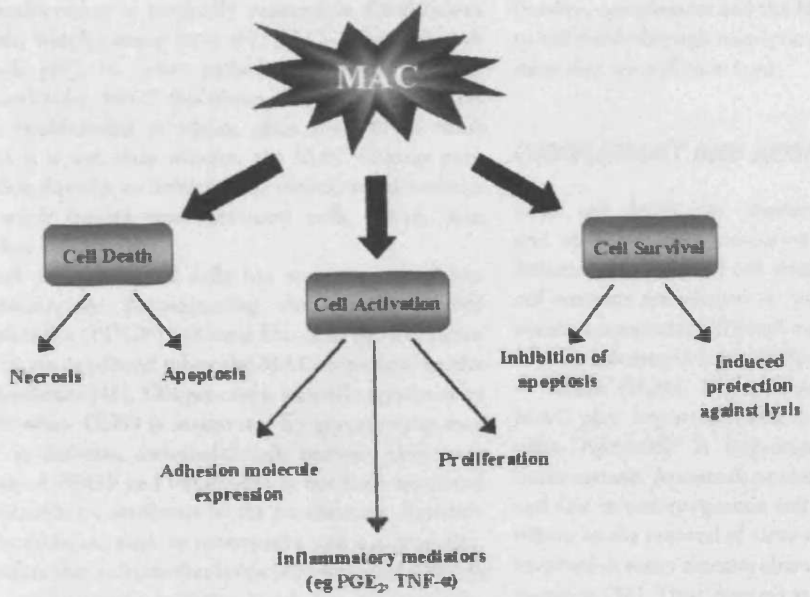

Figure 2 Range of cellular effects of the MAC

The combination of effects observed depends on the cell type and other environmental factors. PGE_2, prostaglandin E_2.

present in non-lytic doses, but contributing to cell death when damage is more extensive [21].

The MAC has been implicated in a variety of other non-lytic effects that differ according to the nature of the target cell and the system interrogated. On phagocytes (neutrophils and macrophages), cell types that are intrinsically resistant to complement-mediated lysis, the MAC induces profound activation, with the production and release of inflammatory mediators such as prostaglandins, thromboxanes, leukotrienes and reactive oxygen species [39]. These processes contribute to homoeostasis by stimulating the inflammatory response and arming the host immune system to deal with invading organisms. On the other hand, these same events may be the cause of much damage. For example, glomerular epithelial cells respond to sublytic MAC levels by the induction of cyclo-oxygenase 2 and the production of prostaglandins that contribute substantially to pathology in membranous glomerulonephritis [40].

From studies *in vitro* on diverse cell types and *in vivo* focusing on inflammatory diseases, much has been learnt about the signal transduction pathways utilized by the MAC. However, the very diversity of cells and systems studied has created a considerable complexity. Numerous signalling pathways for MAC effects, initiated by the activation of calcium flux [37,41], receptor tyrosine kinases [42] and G-proteins [43], have been described. These in turn can activate other signalling cascades in the target cell, including mitogen-activated protein kinase (MAP kinase) and c-Jun N-terminal kinase pathways [44]. Downstream activation of nuclear factor κB (NFκB) and activator protein-1 may result in gene transcription [40]. Current knowledge, although considerable, represents a patchwork of tenuously connected datasets obtained from different targets. No unifying concept of how the MAC triggers activation events has emerged. As a consequence, it is now necessary to step back and critically evaluate the literature in an attempt to identify common pathways of MAC signalling.

Non-lytic effects of the complement MAC are implicated in several diseases. A role in glomerulonephritis was noted above, and recently, dilated cardiomyopathy has come under the spotlight. MAC deposits are found on apparently viable cardiac myocytes in biopsies from patients with this disease, a finding that was correlated with the expression of tumour necrosis factor-α (TNF-α), a powerful pro-inflammatory cytokine and negative inotropic factor [45]. In relevant cell types *in vitro*, TNF-α is induced by the MAC via NFκB, providing a possible explanation for the findings *in vivo*. Many other pathogenic processes in heart disease are being traced to similar non-lytic activating effects of the MAC, including a role in vascular smooth muscle proliferation and remodelling in atherosclerosis [46]. The role of the MAC in the pathological proliferation of cells is now receiving considerable attention.

Complement and cell proliferation

The MAC has been implicated as a stimulus to cell proliferation in a number of scenarios over the past 10 years. *In vivo* models of mesangioproliferative glomerulonephritis, for example, have shown that mesangial

cell proliferation is markedly reduced in C6-deficient animals, which cannot form the MAC, compared with controls [47]. In other pathological states, such as atherosclerosis, MAC deposition occurs in areas where active proliferation is taking place [46]. From these studies it is not clear whether the MAC induces proliferation directly, or indirectly by stimulating the release of growth factors from activated cells, which then stimulate cell division.

Work on endothelial cells has supported the latter explanation by demonstrating that platelet-derived growth factor (PDGF) and basic fibroblast growth factor (bFGF) are produced when the MAC is present on the cell membrane [48]. This process is crucially regulated by CD59: when CD59 is inactivated by glycation, as may occur in diabetes, endothelial cells increase their production of PDGF and bFGF [49]. It has been suggested that this effect contributes to the proliferative disorders seen in diabetes, such as retinopathy and nephropathy. These data also indicate that levels of functional CD59 on a cell surface may be critical not only for protecting the cell, but also in dictating its response to sublytic complement attack.

In studies using aortic smooth muscle cells [50] and Schwann cells [51], non-lethal MAC attack did indeed directly stimulate cell proliferation. Other studies have shown an increase in DNA synthesis in response to the MAC, which in replication-competent fibroblasts probably reflects increased proliferation [52], but in end-cells such as oligodendrocytes [53] and mesangial cells [54] does not. Thus the MAC induces entry to the cell cycle in many cell types, but only takes cells right through to division if they are intrinsically capable of doing so. The MAC can also amplify mitogenic signals from other growth factors, such as PDGF, adding a further level of complexity.

Although the MAC has been clearly implicated in pathological cell proliferation, such as occurs in atherosclerosis, physiologically this response may be important in repairing tissues following inflammation. Thus, while the proliferative effects of the MAC may be damaging in the acute phase of inflammation, during resolution it may assume an important role in repair.

The data summarized above show that the MAC of complement must now be seen in a new light: while the MAC certainly can lyse cells, its non-lytic effects may be of much greater physiological and pathological relevance (summarized in Figure 2). MAC can promote inflammation, increase the resistance of cells to further lytic attack by all sorts of pore formers, and even drive cells to divide. Studies in disease are not only yielding interesting information on how the MAC signals to the cell, but also promising to strongly influence the direction of future therapeutic approaches. Already, it is clear that lytic cell death and the MAC are not related in the simple and straightforward manner that was the accepted dogma. Further, complement and the MAC may even contribute to cell death through non-lytic processes, and it is to this issue that we will now turn.

COMPLEMENT AND APOPTOSIS

Lytic cell death, the 'classical' role of complement and the MAC, is associated with necrosis – a pro-inflammatory form of cell death in tissues whereby the cell contents are released to propagate inflammation. In contrast, apoptotic cell death does not involve the release of cell contents and is considered to be non-inflammatory in nature [55,56]. Nevertheless, complement and the MAC play important roles in regulating apoptosis *in vivo*. Apoptosis is important in development and homoeostasis. Apoptotic processes regulate cell numbers and fate in embryogenesis and tissue remodelling, contribute to the removal of virus-infected cells [57], and are involved in many diseases characterized by abnormal cell turnover [58]. Thus diseases as diverse as AIDS, cancer and neurodegeneration may involve altered apoptotic pathways.

Apoptosis can be defined by morphological criteria: classically, cellular shrinkage, membrane blebbing, nuclear condensation and DNA fragmentation are seen. At the molecular level, the execution of apoptosis centres on a group of enzymes known as the caspases that cleave various proteins in the cell to produce the typical morphological changes [59]. The decision to commit suicide by apoptosis is largely governed by the Bcl-2 family of proteins. These integrate signals from pro-apoptotic and anti-apoptotic stimuli from both the death receptor (e.g. Fas–Fas-ligand) and mitochondrial pathways of induction. They are able to homo- and heterodimerize, and the relative levels of the pro-apoptotic (e.g. Bax) versus the anti-apoptotic (e.g. Bcl-2) members of the family dictate whether or not apoptosis occurs. These signalling proteins act at the level of the mitochondrial membrane to maintain its integrity and regulate the release of cytochrome *c*, which is responsible for triggering the execution caspases such as caspase 3 [59,60].

Complement influences apoptosis at two distinct levels: first, in deciding the fate of the cell, and secondly, in helping phagocytes to dispose of the corpses of apoptosed cells.

Complement in the initiation or inhibition of apoptosis

Several different products of complement influence the decision to proceed to apoptosis. C5a, one of the small anaphylatoxic fragments of complement, inhibits the spontaneous apoptosis of neutrophils, extending their lifespan after recruitment to sites of inflammation [61]. Other inflammatory mediators may facilitate this protective function of C5a; for example, perforin, a pore-

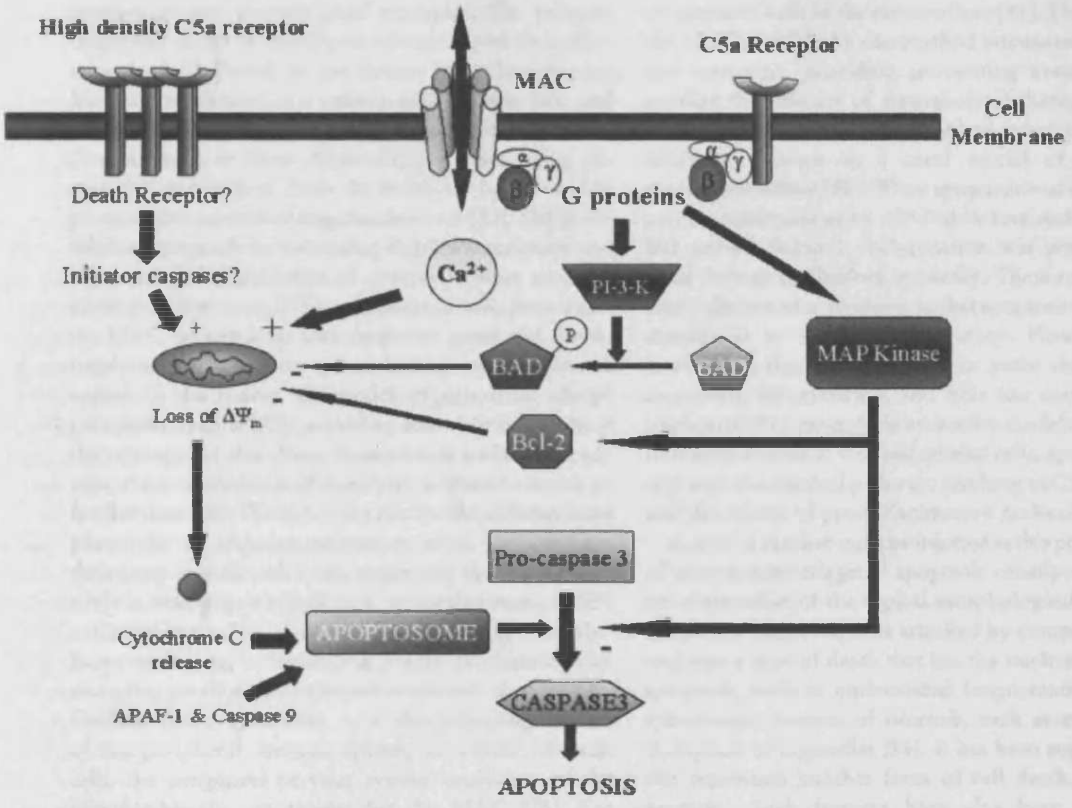

Figure 3 Proposed signalling events involved in the initiation and inhibition of apoptosis by the MAC and C5a

Formation of the MAC results in the influx of calcium. In pro-apoptotic doses, this results in the loss of the mitochondrial transmembrane potential ($\Delta\Psi_m$), leading to the release of cytochrome *c*, which forms the apoptosome with APAF-1 (apoptosis protease-activating factor-1) and caspase 9. Apoptosis is executed by caspase 3. In cells where the MAC inhibits this, G-proteins are activated, and phosphoinositide 3-kinase (PI-3-K) phosphorylates Bad, and/or MAP kinase pathways increase the expression of Bcl-2. These events act to inhibit apoptosis at the level of the mitochondrion. MAP kinase pathways also inhibit the cleavage of pro-caspase 3. The effects of C5a are dependent on the density of the C5a receptor: at low density, apoptosis is inhibited by a G-protein-dependent pathway, whereas at high density, we propose that C5a receptors may act in a fashion analogous to death receptors such as TNF receptors.

forming molecule produced by cytotoxic T cells, can up-regulate the expression of the C5a receptor on neutrophils when present in non-lethal doses [62]. C5a can also protect neurons. Glutamate is an excitatory neurotoxin that triggers the apoptotic death of neurons *in vitro* and is implicated in neuronal death in both Alzheimer's disease and MS [63]. In a model of excitotoxic neuronal death, C5a inhibited apoptosis via inhibition of caspase 3 activity [64]. Confusingly, in a related system, C5a was found to increase the apoptosis of a neuronal cell line [65]. The anaphylatoxin C3a has also been shown to protect neurons *in vitro* from apoptosis induced by the excitotoxic agent *N*-methyl-D-aspartate (NMDA) [66]. Interestingly, in stroke the C3a receptor is up-regulated on neurons, and it is tempting to speculate that this may aid cell survival [67].

C5a plays an important role in sepsis, in which a massive systemic inflammatory response occurs that can result in multi-organ failure and death [68]. One of its features is a catastrophic dysregulation of the immune response. Immunosuppression occurs in part as a result of immune cells undergoing apoptosis, and C5a is implicated as a trigger. Thymocytes undergo intense apoptosis in the caecal ligation and puncture model of sepsis [69,70]. Prior to the onset of apoptosis, thymocytes up-regulate C5a receptor expression in response to lipopolysaccharide and interleukin-6 [69]. When these cells are then exposed to C5a, they undergo apoptosis in a caspase-dependent manner (Figure 3). This does not happen to thymocytes with a normal C5a receptor density, and therefore is only seen in the acute septic situation. Importantly, this effect may be blocked with anti-C5a antibodies, a treatment that is still effective in the model even after the onset of sepsis, when most other therapies are without effect [68,71].

The MAC, among its many non-lytic effects, also plays a role in modulating apoptosis; the proposed signalling pathways are presented in Figure 3. Again, diseases of the

nervous system provide good examples. The primary target cell in MS is the oligodendrocyte, and demyelination is the hallmark of the disease [43]. Complement has been implicated as a pathogenic factor in MS, and MAC-mediated killing of oligodendrocytes has been demonstrated *in vitro*. However, far from being destructive, at non-lytic doses the MAC has been found to promote the survival of oligodendrocytes [72]. The MAC inhibits apoptosis by increasing Bcl-2 transcription and suppressing the activation of caspase 3. More recently, *in vivo* studies using DNA microarrays have shown that the MAC up-regulates anti-apoptotic genes and down-regulates pro-apoptotic genes in the central nervous system in the rodent MS model, experimental allergic encephalomyelitis [73], providing some confirmation of the relevance of this effect. However, as we have already seen, the consequences of non-lytic membrane attack go further than this. The MAC can reverse the differentiated phenotype of oligodendrocytes *in vitro*, and promote their entry into the cell cycle, suggesting that it may have a role in brain repair as well as in promoting survival [53].

Complement has also been implicated in Guillain–Barre syndrome, a devastating illness characterized by ascending paralysis, areflexia and autonomic dysfunction. Guillain–Barre syndrome is a demyelinating disease of the peripheral nervous system, in which Schwann cells, the peripheral nervous system equivalent of the oligodendrocyte, are targets for the MAC [74]. For Schwann cells too, the MAC is anti-apoptotic when present in non-lytic doses, a finding that accords well with its ability to induce proliferation in this cell type, again indicating a role in repair [51,75].

In other circumstances, the MAC has been implicated as a trigger to apoptosis. Complement is an important player in the pathogenesis of a number of renal inflammatory diseases in which substantial deposits of complement components are found in the kidney. Recent studies *in vitro* have shown that exposure of renal mesangial cells to non-lytic amounts of MAC caused the cells to undergo apoptosis [76]. MAC-triggered cell death occurred through a caspase-dependent pathway, specifically via caspase 3, and the cells displayed characteristic features of apoptosis. Evidence from animal models of renal disease also implicates the MAC in triggering apoptosis. In antibody-dependent glomerulonephritis models, such as those induced by concanavalin A or anti-Thy1.1, the abundant glomerular cell apoptosis seen in normal rats was much reduced in C6-deficient rats, in which the MAC cannot form [77,78].

Complement has been implicated in cell death in many models of ischaemia/reperfusion injury, e.g. myocardial infarction and stroke [79,80]. Cell death occurs via apoptosis as well as necrosis in these injuries [81], and the MAC contributes to both. In a myocardial ischaemia/reperfusion model, anti-C5 antibodies inhibited damage following reperfusion and markedly reduced the number of apoptotic cells in the myocardium [81]. Thus blocking the MAC and C5a by this method attenuated both lysis and apoptosis, providing an exciting avenue for improving the efficacy of thrombolytic therapy. Importantly, apoptosis has been found to occur prior to the onset of necrosis in a renal model of ischaemia/reperfusion injury [82]. When apoptosis was inhibited by caspase inhibitors or by survival factors such as insulin-like growth factor-1, inflammation was prevented and renal damage diminished markedly. These results are, at first sight, counter-intuitive, in that apoptotic cell death is considered to be non-inflammatory. However, there is evidence that apoptosis can in some circumstances exacerbate inflammation, and here too complement is implicated. For example, in an *in vitro* model of apoptosis in human umbilical vein endothelial cells, apoptotic cells activated the classical pathway, resulting in C3 deposition and the release of pro-inflammatory molecules [83].

A note of caution must be injected at this point. Studies of complement-triggered apoptosis usually rely heavily on observation of the typical morphological changes of apoptosis. However, cells attacked by complement may undergo a type of death that has the nuclear features of apoptosis, such as nucleosomal fragmentation, but the cytoplasmic features of necrosis, such as swelling and disruption of organelles [84]. It has been suggested that this represents another form of cell death, 'apoptotic necrosis'. Such features have also been noted and investigated *in vitro*, and these studies have shown that such death is primarily necrotic, but with secondary features that resemble nuclear apoptosis, most likely due to an extracellular DNase entering through the disrupted membrane [85]. Studies that identify apoptosis by DNA fragmentation and TUNEL (terminal deoxynucleotidyl UDP nick-end labelling) assay may therefore be misleading.

Complement in the recognition and clearance of apoptotic cells

Complement is also involved in the clearance of apoptotic cells. The safe clearance of cells dying by apoptosis is essential in order to prevent an inflammatory response [55,86]. If apoptotic cells are not cleared efficiently, they undergo secondary necrosis, releasing pro-inflammatory mediators into the environment. Most apoptotic cells are cleared by professional phagocytes such as macrophages, although they can be cleared by other cells, albeit less efficiently. Numerous cell surface features of apoptotic cells have been implicated in their recognition by phagocytes [55,87,88]. The contribution of complement had received surprisingly little attention until quite recently [89]. The surface blebs that are characteristic of cells undergoing apoptosis were shown to bind C1q through the globular head domains, a conformation that permits activation of the classical pathway and deposition of other complement fragments [90]. C1q binds specific

receptors on the phagocyte surface, including CD91 and calreticulin, to initiate phagocytosis [87]. Mannose-binding lectin, the lectin pathway analogue of C1q, also binds apoptotic cells and recruits phagocytes through these same receptors. Apoptotic cells may also activate the alternative pathway directly [83], and all three pathways may result in C3 deposition and activation of the terminal pathway. Down-regulation of membrane complement regulators on cells undergoing apoptosis may also contribute to increased 'opsonization' of the apoptotic cell [91]. Fragments of C4 and C3 deposited on the apoptotic cells will bind the phagocyte-expressed receptors CR1 (complement receptor 1), CR3 and CR4 to further aid recognition and clearance [92]. Binding through CR3 also signals the phagocyte to down-regulate the secretion of interleukin-12 and interferon-γ. This response has an additional anti-inflammatory effect by dampening cell-mediated immunity [93]. From such studies, it has become increasingly clear that phagocytes respond to complement and other apoptotic cell surface signals in the context of their environment, thus defining the 'meaning' of cell death. Phagocytes may respond by altering the susceptibility of neighbouring cells to death, or by modulating the inflammatory response.

The relevance of complement for apoptosis is clearly evident in systemic lupus erythematosus (SLE), a systemic autoimmune disorder characterized by autoantibodies to nuclear antigens [94]. The most obvious role that complement plays in this disease is in causing tissue damage when activated by immune complexes deposited in organs such as the kidneys [94]. This results in inflammation in the kidney and other affected organs. However, complement deficiencies, particularly those of the classical pathway, also predispose to pathology resembling SLE, a finding that appears at first to weaken the case for complement involvement [95,96]. The association with SLE is seen most strikingly in C1q deficiency, the strongest single gene association with SLE, and to a lesser extent with C4 and C2 deficiency [95], and can be explained by the roles noted above for C1q and other complement components in clearing apoptotic cells. This is evident in C1q-deficient mice, where multiple apoptotic bodies were seen in the kidney in association with a glomerulonephritis similar to that seen in SLE [97]. Even in the absence of complement deficiency, SLE is associated with complement consumption and with antibodies against C1q, both of which predict severe disease [98]. This has led to the hypothesis that SLE in humans and mice is caused by defects in the removal of apoptotic cell debris, the 'waste disposal' hypothesis [94]. Apoptotic cells that are not cleared generate an autoimmune response because cytoplasmic and nuclear antigens that are normally sequestered become exposed at the cell surface or outside the lysed cell [89,99,100]. Apoptotic cells undergoing secondary necrosis may also provide 'danger' signals to the antigen-presenting cells and T cells in the area, converting the response to these antigens from tolerogenic into immunogenic [55]. Activated T cells can then stimulate autoreactive B cells to differentiate into plasma cells and start producing autoantibodies [94].

The 'waste disposal' hypothesis is further supported by the observations that mice deficient in serum amyloid P (SAP) or DNase 1, both of which are involved in the clearance of apoptotic debris, also develop an SLE-like disease [101,102]. The pentraxin SAP solubilizes DNA and chromatin in the extracellular fluid and transports it to the liver, where it is catabolized, while DNase1 breaks down DNA into non-antigenic fragments. Indeed, SLE patients have low levels of DNase 1, adding support to the involvement of this enzyme, and to the importance of reducing the antigenicity of DNA by cleaving it [102]. C-reactive protein, also a member of the pentraxin family and closely related to SAP, binds small nuclear ribonucleoproteins. These are also autoantigens in SLE, and it is widely recognized that disease flares are associated with a deficient C-reactive protein response, which may be associated with poor clearance of such autoantigenic material [103].

These studies emphasize the multi-factorial process of efficient waste disposal, and highlight the fact that defects in any one of the key handling processes can cause failed clearance and pathology. They also suggest that treatment strategies targeting the clearance mechanisms for apoptotic cells might be effective in many diseases.

CONCLUSIONS AND FUTURE DIRECTIONS

We are now at a fascinating point in our understanding of the roles of complement in homoeostasis and pathology. The 'classical' role of complement as a lytic system remains important, but it is now clear that complement and the MAC have a range of non-lethal effects on cells, acting as a drive to inflammation, but also involved in the induction of proliferation and resistance to killing by both lysis and apoptosis [18,72]. In other circumstances, complement can kill by inducing apoptosis [76], and there is abundant evidence for a role in the efficient clearance of apoptotic cell debris [87]. There are several situations where complement appears to be playing opposing roles in the same system: both promoting cell survival and inducing apoptosis; both causing cell lysis and inducing protection against lysis. These apparent contradictions probably arise due to the artificial systems in which most have been demonstrated, but do illustrate the protean effects of this superficially simple system in complex tissues.

Perhaps the first step towards clarification is to recognize that complement has these diverse activities. Current complement therapies aim to inhibit complement activation in order to control inflammation [3].

What is clear from the above discussion is that it may not always be to the benefit of the patient for complement to be inhibited. The risks relating to infection are well rehearsed, but a loss of the 'non-classical' complement activities described above might be of more relevance to the patient. To overcome these problems, complement inhibitors may have to be carefully tailored and targeted to act only in those areas where complement activation needs to be controlled. Ultimately, we need to understand not only how complement influences cell fate, but also how we can alter this when it goes awry in disease.

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REFERENCES

1 Morgan, B. P. (1990) Complement: Clinical Aspects and Relevance to Disease, Academic Press, London
2 Ehrlich, P. and Morgenroth, J. (1899) Zur theorie der lysinwirkung. Berlin Klin. Wochenchr. 36, 6–9
3 Kirschfink, M. (2001) Targeting complement in therapy. Immunol. Rev. 180, 177–189
4 Walport, M. J. (2001) Complement. N. Engl. J. Med. 344, 1058–1066
5 Barrington, R., Zhang, M., Fischer, M. and Carroll, M. C. (2001) The role of complement in inflammation and adaptive immunity. Immunol. Rev. 180, 5–15
6 Podack, E. R. and Tschopp, J. (1984) Membrane attack by complement. Mol. Immunol. 21, 589–603
7 Morgan, B. P. and Harris, C. L. (1999) Complement Regulatory Proteins, Academic Press, London
8 Linton, S. M. and Morgan, B. P. (1999) Complement activation and inhibition in experimental models of arthritis. Mol. Immunol. 36, 905–914
9 Welch, T. R. (2002) Complement in glomerulonephritis. Nat. Genet. 31, 333–334
10 Storch, M. K., Piddlesden, S., Haltia, M., Iivanainen, M., Morgan, P. and Lassmann, H. (1998) Multiple sclerosis: in situ evidence for antibody- and complement-mediated demyelination. Ann. Neurol. 43, 465–471
11 Ffrench-Constant, C. (1994) Pathogenesis of multiple sclerosis. Lancet 343, 271–275
12 Bordet, J. (1900) Les serums haemolytiques, leurs antitoxines et les théories des serum cytolytiques. Ann. Inst. Pasteur 15, 257–270
13 Bhakdi, S. and Tranum-Jensen, J. (1991) Complement lysis: a hole is a hole. Immunol. Today 12, 318–320
14 Esser, A. F. (1991) Big MAC attack: complement proteins cause leaky patches. Immunol. Today 12, 316–318
15 Porcel, J. M., Peakman, M., Senaldi, G. and Vergani, D. (1993) Methods for assessing complement activation in the clinical immunology laboratory. J. Immunol. Methods 157, 1–9
16 Koski, C. L., Ramm, L. E., Hammer, C. H., Mayer, M. M. and Shin, M. L. (1983) Cytolysis of nucleated cells by complement: cell death displays multi-hit characteristics. Proc. Natl. Acad. Sci. U.S.A. 80, 3816–3820
17 Campbell, A. K., Patel, A., Houston, W. A. et al. (1989) Photoproteins as indicators of intracellular free Ca^{2+}. J. Biolumin. Chemilumin. 4, 463–474
18 Morgan, B. P. (1989) Complement membrane attack on nucleated cells: resistance, recovery and non-lethal effects. Biochem. J. 264, 1–14
19 Campbell, A. K., Daw, R. A., Hallett, M. B. and 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, 551–560
20 Scolding, N. J., Morgan, B. P., Houston, W. A., Linington, C., Campbell, A. K. and Compston, D. A. (1989) Vesicular removal by oligodendrocytes of membrane attack complexes formed by activated complement. Nature (London) 339, 620–622
21 Morgan, B. P., Luzio, J. P. and Campbell, A. K. (1986) Intracellular Ca^{2+} and cell injury: a paradoxical role of Ca^{2+} in complement membrane attack. Cell Calcium 7, 399–411
22 Sala-Newby, G. B., Taylor, K. M., Badminton, M. N., Rembold, C. M. and Campbell, A. K. (1998) Imaging bioluminescent indicators shows Ca^{2+} and ATP permeability thresholds in live cells attacked by complement. Immunology 93, 601–609
23 Kim, S. H., Carney, D. F., Hammer, C. H. and Shin, M. L. (1987) Nucleated cell killing by complement: effects of C5b-9 channel size and extracellular Ca^{2+} on the lytic process. J. Immunol. 138, 1530–1536
24 Papadimitriou, J. C., Phelps, P. C., Shin, M. L., Smith, M. W. and Trump, B. F. (1994) Effects of Ca2+ deregulation on mitochondrial membrane potential and cell viability in nucleated cells following lytic complement attack. Cell Calcium 15, 217–227
25 Papadimitriou, J. C., Ramm, L. E., Drachenberg, C. B., Trump, B. F. and Shin, M. L. (1991) Quantitative analysis of adenine nucleotides during the prelytic phase of cell death mediated by C5b-9. J. Immunol. 147, 212–217
26 Bhakdi, S., Kuller, G., Muhly, M., Fromm, S., Seibert, G. and Parrisius, J. (1987) Formation of transmural complement pores in serum-sensitive Escherichia coli. Infect. Immun. 55, 206–210
27 Born, J. and Bhakdi, S. (1986) Does complement kill E. coli by producing transmural pores? Immunology 59, 139–145
28 Morgan, B. P. and Walport, M. J. (1991) Complement deficiency and disease. Immunol. Today 12, 301–306
29 Meri, S., Morgan, B. P., Davies, A. et al. (1990) Human protectin (CD59), an 18,000–20,000 MW complement lysis restricting factor, inhibits C5b-8 catalysed insertion of C9 into lipid bilayers. Immunology 71, 1–9
30 Jurianz, K., Ziegler, S., Garcia-Schuler, H. et al. (1999) Complement resistance of tumor cells: basal and induced mechanisms. Mol. Immunol. 36, 929–939
31 Reiter, Y., Ciobotariu, A. and Fishelson, Z. (1992) Sublytic complement attack protects tumor cells from lytic doses of antibody and complement. Eur. J. Immunol. 22, 1207–1213
32 Reiter, Y. and Fishelson, Z. (1992) Complement membrane attack complexes induce in human leukemic cells rapid expression of large proteins (L-CIP). Mol. Immunol. 29, 771–781
33 Fishelson, Z., Hochman, I., Greene, L. E. and Eisenberg, E. (2001) Contribution of heat shock proteins to cell protection from complement-mediated lysis. Int. Immunol. 13, 983–991
34 Reiter, Y., Ciobotariu, A., Jones, J., Morgan, B. P. and Fishelson, Z. (1995) Complement membrane attack complex, perforin, and bacterial exotoxins induce in K562 cells calcium-dependent cross-protection from lysis. J. Immunol. 155, 2203–2210
35 Morgan, B. P., Dankert, J. R. and Esser, A. F. (1987) Recovery of human neutrophils from complement attack: removal of the membrane attack complex by endocytosis and exocytosis. J. Immunol. 138, 246–253
36 Carney, D. F., Koski, C. L. and Shin, M. L. (1985) Elimination of terminal complement intermediates from the plasma membrane of nucleated cells: the rate of disappearance differs for cells carrying C5b-7 or C5b-8 or a mixture of C5b-8 with a limited number of C5b-9. J. Immunol. 134, 1804–1809
37 Kraus, S. and Fishelson, Z. (2000) Cell desensitization by sublytic C5b-9 complexes and calcium ionophores depends on activation of protein kinase C. Eur. J. Immunol. 30, 1272–1280

38 Morgan, B. P. and Campbell, A. K. (1985) The recovery of human polymorphonuclear leucocytes from sublytic complement attack is mediated by changes in intracellular free calcium. Biochem. J. 231, 205–208
39 Hansch, G. M., Seitz, M., Martinotti, G., Betz, M., Rauterberg, E. W. and Gemsa, D. (1984) Macrophages release arachidonic acid, prostaglandin E2, and thromboxane in response to late complement components. J. Immunol. 133, 2145–2150
40 Takano, T., Cybulsky, A. V., Yang, X. and Aoudjit, L. (2001) Complement C5b-9 induces cyclooxygenase-2 gene transcription in glomerular epithelial cells. Am. J. Physiol. Renal Physiol. 281, F841–F850
41 Cybulsky, A. V., Bonventre, J. V., Quigg, R. J., Lieberthal, W. and Salant, D. J. (1990) Cytosolic calcium and protein kinase C reduce complement-mediated glomerular epithelial injury. Kidney Int. 38, 803–811
42 Cybulsky, A. V., Takano, T., Papillon, J. and McTavish, A. J. (1999) Complement C5b-9 induces receptor tyrosine kinase transactivation in glomerular epithelial cells. Am. J. Pathol. 155, 1701–1711
43 Rus, H. G., Niculescu, F. I. and Shin, M. L. (2001) Role of the C5b-9 complement complex in cell cycle and apoptosis. Immunol. Rev. 180, 49–55
44 Peng, H., Takano, T., Papillon, J., Bijian, K., Khadir, A. and Cybulsky, A. V. (2002) Complement activates the c-Jun N-terminal kinase/stress-activated protein kinase in glomerular epithelial cells. J. Immunol. 169, 2594–2601
45 Zwaka, T. P., Manolov, D., Ozdemir, C. et al. (2002) Complement and dilated cardiomyopathy: a role of sublytic terminal complement complex-induced tumor necrosis factor-α synthesis in cardiac myocytes. Am. J. Pathol. 161, 449–457
46 Niculescu, F. and Rus, H. (1999) Complement activation and atherosclerosis. Mol. Immunol. 36, 949–955
47 Brandt, J., Pippin, J., Schulze, M. et al. (1996) Role of the complement membrane attack complex (C5b-9) in mediating experimental mesangioproliferative glomerulonephritis. Kidney Int. 49, 335–343
48 Benzaquen, L. R., Nicholson-Weller, A. and Halperin, J. A. (1994) Terminal complement proteins C5b-9 release basic fibroblast growth factor and platelet-derived growth factor from endothelial cells. J. Exp. Med. 179, 985–992
49 Acosta, J., Hettinga, J., Fluckiger, R. et al. (2000) Molecular basis for a link between complement and the vascular complications of diabetes. Proc. Natl. Acad. Sci. U.S.A. 97, 5450–5455
50 Niculescu, F., Badea, T. and Rus, H. (1999) Sublytic C5b-9 induces proliferation of human aortic smooth muscle cells: role of mitogen activated protein kinase and phosphatidylinositol 3-kinase. Atherosclerosis 142, 47–56
51 Dashiell, S. M., Rus, H. and Koski, C. L. (2000) Terminal complement complexes concomitantly stimulate proliferation and rescue of Schwann cells from apoptosis. Glia 30, 187–198
52 Halperin, J. A., Taratuska, A. and Nicholson-Weller, A. (1993) Terminal complement complex C5b-9 stimulates mitogenesis in 3T3 cells. J. Clin. Invest. 91, 1974–1978
53 Rus, H. G., Niculescu, F. and Shin, M. L. (1996) Sublytic complement attack induces cell cycle in oligodendrocytes. J. Immunol. 156, 4892–4900
54 Couser, W. G., Pippin, J. W. and Shankland, S. J. (2001) Complement (C5b-9) induces DNA synthesis in rat mesangial cells in vitro. Kidney Int. 59, 905–912
55 Savill, J. and Fadok, V. (2000) Corpse clearance defines the meaning of cell death. Nature (London) 407, 784–788
56 Voll, R. E., Herrmann, M., Roth, E. A., Stach, C., Kalden, J. R. and Girkontaite, I. (1997) Immunosuppressive effects of apoptotic cells. Nature (London) 390, 350–351
57 Restifo, N. P. (2000) Building better vaccines: how apoptotic cell death can induce inflammation and activate innate and adaptive immunity. Curr. Opin. Immunol. 12, 597–603
58 Thompson, C. B. (1995) Apoptosis in the pathogenesis and treatment of disease. Science 267, 1456–1462
59 Hengartner, M. O. (2000) The biochemistry of apoptosis. Nature (London) 407, 770–776
60 Green, D. R. and Reed, J. C. (1998) Mitochondria and apoptosis. Science 281, 1309–1312
61 Lee, A., Whyte, M. K. and Haslett, C. (1993) Inhibition of apoptosis and prolongation of neutrophil functional longevity by inflammatory mediators. J. Leukocyte Biol. 54, 283–288
62 Hopkins, J. I., Jones, J. and Morgan, B. P. (1998) Non-lethal effects of perforin on polymorphonuclear leukocytes. Biochem. Soc. Trans. 26, S50
63 Pitt, D., Werner, P. and Raine, C. S. (2000) Glutamate excitotoxicity in a model of multiple sclerosis. Nat. Med. (N.Y.) 6, 67–70
64 Mukherjee, P. and Pasinetti, G. M. (2001) Complement anaphylatoxin C5a neuroprotects through mitogen-activated protein kinase-dependent inhibition of caspase 3. J. Neurochem. 77, 43–49
65 Farkas, I., Baranyi, L., Liposits, Z. S., Yamamoto, T. and Okada, H. (1998) Complement C5a anaphylatoxin fragment causes apoptosis in TGW neuroblastoma cells. Neuroscience 86, 903–911
66 van Beek, J., Nicole, O., Ali, C. et al. (2001) Complement anaphylatoxin C3a is selectively protective against NMDA-induced neuronal cell death. Neuroreport 12, 289–293
67 Van Beek, J., Bernaudin, M., Petit, E. et al. (2000) Expression of receptors for complement anaphylatoxins C3a and C5a following permanent focal cerebral ischemia in the mouse. Exp. Neurol. 161, 373–382
68 Huber-Lang, M., Sarma, V. J., Lu, K. T. et al. (2001) Role of C5a in multiorgan failure during sepsis. J. Immunol. 166, 1193–1199
69 Riedemann, N. C., Guo, R. F., Laudes, I. J. et al. (2002) C5a receptor and thymocyte apoptosis in sepsis. FASEB J. 16, 887–888
70 Guo, R. F., Huber-Lang, M., Wang, X. et al. (2000) Protective effects of anti-C5a in sepsis-induced thymocyte apoptosis. J. Clin. Invest. 106, 1271–1280
71 Huber-Lang, M. S., Sarma, J. V., McGuire, S. R. et al. (2001) Protective effects of anti-C5a peptide antibodies in experimental sepsis. FASEB J. 15, 568–570
72 Soane, L., Rus, H., Niculescu, F. and Shin, M. L. (1999) Inhibition of oligodendrocyte apoptosis by sublytic C5b-9 is associated with enhanced synthesis of bcl-2 and mediated by inhibition of caspase-3 activation. J. Immunol. 163, 6132–6138
73 Rus, H., Weerth, S., Sloane, L. et al. (2002) The effect of terminal complement complex on apoptosis gene expression in experimental autoimmune encephalomyelitis. Int. Pharmacol. 2, 1347
74 Putzu, G. A., Figarella-Branger, D., Bouvier-Labit, C., Liprandi, A., Bianco, N. and Pellissier, J. F. (2000) Immunohistochemical localization of cytokines, C5b-9 and ICAM-1 in peripheral nerve of Guillain-Barre syndrome. J. Neurol. Sci. 174, 16–21
75 Hila, S., Soane, L. and Koski, C. L. (2001) Sublytic C5b-9-stimulated Schwann cell survival through PI 3-kinase-mediated phosphorylation of BAD. Glia 36, 58–67
76 Nauta, A. J., Daha, M. R., Tijsma, O., van de Water, B., Tedesco, F. and Roos, A. (2002) The membrane attack complex of complement induces caspase activation and apoptosis. Eur. J. Immunol. 32, 783–792
77 Hughes, J., Nangaku, M., Alpers, C. E., Shankland, S. J., Couser, W. G. and Johnson, R. J. (2000) C5b-9 membrane attack complex mediates endothelial cell apoptosis in experimental glomerulonephritis. Am. J. Physiol. Renal Physiol. 278, F747–F757
78 Sato, T., Van Dixhoorn, M. G., Prins, F. A. et al. (1999) The terminal sequence of complement plays an essential role in antibody-mediated renal cell apoptosis. J. Am. Soc. Nephrol. 10, 1242–1252
79 D'Ambrosio, A. L., Pinsky, D. J. and Connolly, E. S. (2001) The role of the complement cascade in ischemia/reperfusion injury: implications for neuroprotection. Mol. Med. 7, 367–382

80 Monsinjon, T., Richard, V. and Fontaine, M. (2001) Complement and its implications in cardiac ischemia/reperfusion: strategies to inhibit complement. Fundam. Clin. Pharmacol. **15**, 293–306
81 Vakeva, A. P., Agah, A., Rollins, S. A., Matis, L. A., Li, L. and Stahl, G. L. (1998) Myocardial infarction and apoptosis after myocardial ischemia and reperfusion: role of the terminal complement components and inhibition by anti-C5 therapy. Circulation **97**, 2259–2267
82 Daemen, M. A., van 't Veer, C., Denecker, G. et al. (1999) Inhibition of apoptosis induced by ischemia-reperfusion prevents inflammation. J. Clin. Invest. **104**, 541–549
83 Tsuji, S., Kaji, K. and Nagasawa, S. (1994) Activation of the alternative pathway of human complement by apoptotic human umbilical vein endothelial cells. J. Biochem. (Tokyo) **116**, 794–800
84 Shimizu, A., Masuda, Y., Kitamura, H. et al. (2000) Complement-mediated killing of mesangial cells in experimental glomerulonephritis: cell death by a combination of apoptosis and necrosis. Nephron **86**, 152–160
85 Cragg, M. S., Howatt, W. J., Bloodworth, L., Anderson, V. A., Morgan, B. P. and Glennie, M. J. (2000) Complement mediated cell death is associated with DNA fragmentation. Cell Death Differ. **7**, 48–58
86 Fadok, V. A., Bratton, D. L., Konowal, A., Freed, P. W., Westcott, J. Y. and Henson, P. M. (1998) Macrophages that have ingested apoptotic cells in vitro inhibit proinflammatory cytokine production through autocrine/paracrine mechanisms involving TGF-β, PGE2, and PAF. J. Clin. Invest. **101**, 890–898
87 Ogden, C. A., deCathelineau, A., Hoffmann, P. R. et al. (2001) C1q and mannose binding lectin engagement of cell surface calreticulin and CD91 initiates macropinocytosis and uptake of apoptotic cells. J. Exp. Med. **194**, 781–795
88 Nauta, A. J., Raaschou-Jensen, N., Garred, P. et al. (2002) Mannose-binding lectin (MBL) tags apoptotic cells and cell blebs as well as necrotic cells. Int. Pharmacol. **2**, 1348
89 Fishelson, Z., Attali, G. and Mevorach, D. (2001) Complement and apoptosis. Mol. Immunol. **38**, 207–219
90 Korb, L. C. and Ahearn, J. M. (1997) C1q binds directly and specifically to surface blebs of apoptotic human keratinocytes: complement deficiency and systemic lupus erythematosus revisited. J. Immunol. **158**, 4525–4528
91 Jones, J. and Morgan, B. P. (1995) Apoptosis is associated with reduced expression of complement regulatory molecules, adhesion molecules and other receptors on polymorphonuclear leucocytes: functional relevance and role in inflammation. Immunology **86**, 651–660
92 Takizawa, F., Tsuji, S. and Nagasawa, S. (1996) Enhancement of macrophage phagocytosis upon iC3b deposition on apoptotic cells. FEBS Lett. **397**, 269–272
93 Marth, T. and Kelsall, B. L. (1997) Regulation of interleukin-12 by complement receptor 3 signaling. J. Exp. Med. **185**, 1987–1995
94 Walport, M. J. (2001) Complement. N. Engl. J. Med. **344**, 1140–1144
95 Taylor, P. R., Carugati, A., Fadok, V. A. et al. (2000) A hierarchical role for classical pathway complement proteins in the clearance of apoptotic cells in vivo. J. Exp. Med. **192**, 359–366
96 Botto, M. (2001) Links between complement deficiency and apoptosis. Arthritis Res. **3**, 207–210
97 Botto, M., Dell'Agnola, C., Bygrave, A. E. et al. (1998) Homozygous C1q deficiency causes glomerulonephritis associated with multiple apoptotic bodies. Nat. Genet. **19**, 56–59
98 Moroni, G., Trendelenburg, M., Del Papa, N. et al. (2001) Anti-C1q antibodies may help in diagnosing a renal flare in lupus nephritis. Am. J. Kidney Dis. **37**, 490–498
99 Bell, D. A. and Morrison, B. (1991) The spontaneous apoptotic cell death of normal human lymphocytes in vitro: the release of, and immunoproliferative response to, nucleosomes in vitro. Clin. Immunol. Immunopathol. **60**, 13–26
100 Mohan, C., Adams, S., Stanik, V. and Datta, S. K. (1993) Nucleosome: a major immunogen for pathogenic autoantibody-inducing T cells of lupus. J. Exp. Med. **177**, 1367–1381
101 Bickerstaff, M. C., Botto, M., Hutchinson, W. L. et al. (1999) Serum amyloid P component controls chromatin degradation and prevents antinuclear autoimmunity. Nat. Med. (N.Y.) **5**, 694–697
102 Napirei, M., Karsunky, H., Zevnik, B., Stephan, H., Mannherz, H. G. and Moroy, T. (2000) Features of systemic lupus erythematosus in Dnase1-deficient mice. Nat. Genet. **25**, 177–181
103 Gershov, D., Kim, S., Brot, N. and Elkon, K. B. (2000) C-Reactive protein binds to apoptotic cells, protects the cells from assembly of the terminal complement components, and sustains an antiinflammatory innate immune response: implications for systemic autoimmunity. J. Exp. Med. **192**, 1353–1364

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Complement regulator loss on apoptotic neuronal cells causes increased complement activation and promotes both phagocytosis and cell lysis

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Abstract

In neuroinflammatory disease complement (C) activation and neuronal apoptosis occur in areas of active pathology. C has a role in clearing apoptotic debris, but is also known to cause necrotic cell death by insertion of the membrane attack complex (MAC). It is therefore unclear whether C is protective or injurious in this context. Here we examine C regulator expression and susceptibility to C activation, lysis and phagocytosis in human neuronal cells undergoing apoptosis in order to model the in vivo situation. We demonstrate that apoptotic neuronal lines lose the C regulators CD46 and CD59. Regulator loss occurred only on cells positive for apoptotic markers, and was caspase dependent. Both CD46 and CD59 were shed from cells, CD46 as a soluble form following MMP cleavage, and CD59 on apoptotic blebs and as a soluble form. Apoptotic cells activated C and were opsonised more readily than control cells; as a consequence they were more readily phagocytosed by macrophages than non-apoptotic cells. Susceptibility to C-mediated lysis was complicated in that early cells were more sensitive while late apoptotic cells were more resistant to killing. MMP inhibition protected against the increased lysis seen in early apoptotic cells, but had no effect on susceptibility of non-apoptotic cells to C-mediated lysis. Our studies suggest that C activation on apoptotic neuronal cells is delicately balanced between enhancing their safe disposal through phagocytosis, and triggering necrosis by C-mediated lysis. The data suggest that therapeutic MMP inhibition, by restricting loss of CD46, may limit neuronal damage in neurological disease.

Keywords: Complement; Apoptosis; Neuropathology; Phagocytosis; Matrix metalloprotease

1. Introduction

Complement (C) activation occurs in areas of active disease in both neuroinflammation and neurodegeneration (Compston et al., 1989; Emmerling et al., 2000; Mead et al., 2002). Loss of neurons is recognised as a significant component in the pathology of these diseases and in multiple sclerosis (MS) neuronal loss has been correlated with poor recovery of function and permanent neurological disability (Davie et al., 1995). In vitro studies have shown that neurons spontaneously activate C and are particularly susceptible to lytic damage by the membrane attack complex (MAC) (Gasque et al., 1996; Singhrao et al., 2000). It is now becoming evident that neuronal death in MS and experimental autoimmune encephalomyelitis (EAE) is largely apoptotic rather than necrotic, and in EAE, neuronal death via apoptosis has been shown to correlate with functional deficit (Meyer et al., 2001).

Apoptotic cells in the CNS are cleared by microglia or astrocytes in a non-inflammatory manner (Stolzing and Grune, 2004). Failure to clear such apoptotic debris may provoke a damaging inflammatory reaction and exacerbate pathology, since dying cells eventually undergo secondary necrosis and release pro-inflammatory mediators into the environment. Recognition in apoptosis is mediated by changes in molecules expressed at the surface of the dying cell, such as exposure of phosphatidylserine (Li et al., 2003). C has been shown to play a role in clearing apoptotic debris in many tissues through binding of C1q with classical pathway activation and deposition of C3b (Mevorach et al., 1998; Taylor et al., 2000). C activation occurs late in the apoptotic process in most cells suggesting that it may act as a fail-safe mechanism for clearance (Gaipl et al., 2001). However, C is also known to be pro-inflammatory and to cause necrotic cell

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death by insertion of the MAC (Koski et al., 1983). The balance between the clearance enhancing anti-inflammatory properties and necrosis-inducing pro-inflammatory actions of C may be crucial in determining the consequences of apoptotic cell death in tissues.

Self-cells are normally protected from C activation by the membrane C regulators (CReg) CD46 and CD55 controlling the C3 convertase, and CD59 controlling the MAC. Previous work in our laboratory has shown that CD55 and CD59 are lost during neutrophil apoptosis in vitro, and this makes cells more susceptible to C-mediated lysis (Jones and Morgan, 1995).

Such changes in CReg, if they also occur on other cell types in vivo, may be important under pathological conditions. For example, loss of CReg would leave dying neurons stripped of protection against C, and, coupled with their capacity to spontaneously activate C (Singhrao et al., 2000), apoptotic neurons could become potent catalysts for uncontrolled C activation and increasingly prone to C-mediated lysis. However, increased C activation and deposition of C3 fragments could also result in brisk opsonisation and subsequent clearance by phagocytes, avoiding any worsening of the inflammatory response. The timing and regulation of C activation may therefore be critical in deciding the fate of apoptotic neurons and in determining whether inflammation is provoked or inhibited in the brain.

In view of the largely protective responses normally associated with apoptosis, we hypothesised that C activation on apoptotic neurons would be predominantly anti-inflammatory. Our aims were therefore to identify the factors that influence C activation on apoptotic neuronal cells, and study the consequences for the cell, either causing lysis and exacerbating inflammation, or promoting phagocytosis to remove debris efficiently.

2. Materials and methods

2.1. Cells and reagents

IMR-32 neuroblastoma cells (ATCC, Teddington, UK) were maintained in RPMI 1640 (Gibco, Paisley, UK) with 5% fetal calf serum (FCS), sodium pyruvate (5 mM), streptomycin (50 U/ml), penicillin (50 U/ml) and L-glutamine (2 mM). Cells were split and medium replaced every 2–3 days. GM6001, MMP-2, -3, -8, -9 inhibitors and TIMP-3 were from Calbiochem (Nottingham, UK). The caspase inhibitors zVAD-fmk (pan-caspase) and zDEVD-fmk (caspase-3) were from R&D Systems (Abingdon, UK). The mAb BRIC229 (anti-CD59) and BRIC216 (anti-CD55) were from IBGRL (Bristol, UK); MEM258 (anti-CD46) was a kind gift from Dr. V. Horesji, Prague. B7 (anti-C9 neoepitope) was raised in-house; C3/30 (anti-C3b) was from Dr. P. Taylor (Ciba, Horsham, UK). Donkey anti-mouse IgG-horseradish peroxidase (HRPO) was from BioRad (Hemel Hempstead, UK). Sodium pyruvate, trichloroacetic acid (TCA), PKH26 mini linker kit and Accuspin tubes were obtained from Sigma (Gillingham, UK). C fixation diluent (CFD) tablets were from Oxoid (Basingstoke, UK). C8 depleted (C8d) human serum was prepared using an anti-C8 affinity column as described (Morgan, 2000). Heat inactivated normal human serum (HI-NHS) was prepared by heating NHS to 56 °C for 45 min in a water bath. Lysis buffer contained 150 mM NaCl, 50 mM Tris, 5 mM EDTA, 1% NP-40 and aprotinin (10 μg/ml; Sigma).

2.2. Flow cytometry and apoptosis assays

IMR-32 were plated out and allowed to adhere for 24 h. Induction of apoptosis was achieved by replacing the medium with fresh medium containing 1 μM camptothecin (CPT; Sigma), or 60 μM each of C2 and C6 ceramide (Biomol; Affiniti Research Products, Exeter, UK). Cells were also incubated with appropriate carrier controls (either DMSO diluted 1 in 10,000 or 1% BSA, respectively). For UV treatment, cells were irradiated with UV (400 mJ) in serum-free RPMI. Non-adherent cells were harvested by removing the medium; adherent cells were released by brief trypsinisation and washing in RPMI. For total cell collection, adherent and non-adherent cells were pooled prior to centrifugation. Where MMPs were inhibited, this was only during apoptosis induction, and cells were washed three times before subsequent steps in the experiment (C deposition or lysis assays).

For annexin-V-FITC (BD Biosciences, Oxford, UK) staining, 10^5 cells were washed in annexin binding buffer (ABB; 10 mM HEPES, 140 mM NaCl, 2.5 mM $CaCl_2$, pH 7.4) and incubated with annexin-V-FITC according to the manufacturer's instructions, with 2 μg/ml propidium iodide (PI) for 20 min at 22 °C in the dark. For immunostaining, primary antibodies were incubated with 10^5 cells in FACS buffer (100 μl; PBS, 1% BSA, 5 mM EDTA) for 30 min at 22 °C, followed by RPE-conjugated goat anti-mouse IgG (Dakocytomation, Cambridgeshire, UK) at 22 °C in the dark for 30 min. All analysis was carried out on a BD FACScalibur flow cytometer.

2.3. Complement deposition assays

C1q-FITC and BSA-FITC were made by direct conjugation as described (Roederer, 2004). CPT treated and control IMR-32 were harvested and 10^5 cells incubated with 100 μl NHS (5%), C8d (20%) or with C1q-FITC (10 μg/ml) in RPMI for 1 h at 37 °C. Appropriate heat-inactivated serum controls were used. Cells stained with C1q-FITC were washed in FACS buffer and analysed on the flow cytometer on the FL-1 channel. Cells incubated with C8d were incubated with anti-C3b mAb (C3/30 5 μg/ml) in FACS buffer on ice for 30 min. To assess MAC binding, NHS treated cells were stained with B7 (5 μg/ml) in FACS buffer on ice for 30 min. Following washing, cells were incubated with anti-mouse IgG-RPE in FACS buffer for 30 min on ice in the dark and analysed on a flow cytometer. For double staining experiments, cells were then stained for annexin-V-FITC.

2.4. Isolation of apoptotic blebs from culture supernatant

Apoptosis was induced in IMR-32 by incubation with CPT as described above. Cells and culture supernatant were harvested. Harvested cells were lysed on ice for 30 min. The lysate was then spun at 16,000 × *g* at 4 °C for 15 min, and the pellet

discarded. The lysate supernatant was frozen at −20 °C until analysis.

The cell-free culture supernatant was concentrated 10 times in an Amicon ultracentrifugation system with a 10 kDa cut-off filter at 4 °C. The cell-free supernatant was then spun at 100,000 × *g* for 1 h at 4 °C in a Beckman TL-100 ultracentrifuge. The supernatant was collected, and the pellet dissolved in 1× SDS-PAGE loading buffer, boiled, and then frozen at −20 °C until analysis. Proteins remaining in the supernatant were precipitated with 10% TCA for 30 min on ice. The samples were then spun at 16,000 × *g* at 4 °C for 10 min, and the pellet was washed twice in acetone and spun at 16,000 × *g* for 5 min at 4 °C before discarding the supernatant and vacuum drying the pellet. The pellet was then dissolved in 1× SDS-PAGE loading buffer, boiled and frozen at −20 °C until analysis.

Cell lysates, culture supernatant and ultracentrifugation pellets were subjected to SDS-PAGE and Western blotting was carried out using primary antibodies BRIC229 and MEM258 and secondary antibody donkey anti-mouse IgG-HRPO. Membranes were developed using the ECL Western Blotting detection system (Pierce) and imaged using Kodak Medical Imaging film.

2.5. C lysis assays

NHS or HI-NHS were diluted in CFD, added to 10^5 cells and incubated for 1 h at 37 °C. Triton X-100 (0.1%) in CFD was used as the 100% lysis control and CFD alone was used for the 0% lysis control. Following incubation, 50 μl of sample supernatant was taken for LDH assay. For the LDH assay, solution A was 5.6 mM Tris, 5.6 mM EDTA, pH 7.4, solution B was sodium pyruvate (14.85 mg/ml) and solution C was NADH (1.3 mg/ml) in solution A made up fresh just before use. To each well of the 96-well plate 100 μl solution C was added. Just before analysis, 10 μl solution B was added to each well. Absorbance readings were taken at 340 nm every 2 min for 20 min on a FLUOstar Optima spectrophotometer (BMG Labtechnologies, Aylesbury, UK). The rate of fall of absorbance at 340 nm was calculated for each well, reflecting the activity of LDH present, and the following equation applied:

$$\%\text{C-specific lysis} = \frac{\text{NHS lysis} - \text{HI-NHS lysis}}{\text{100\% lysis} - \text{HI-NHS lysis}} \times 100$$

For the PI lysis assay, serum was diluted in RPMI and incubated with cells. After 1 h, cells were stained with PI (2 μg/ml) and analysed on a flow cytometer. Percent lysis was calculated as above.

2.6. Phagocytosis assay

PBMCs were isolated from buffy coat (Welsh Blood Transfusion Service Llantrisant, UK) using lymphoprep (Axis-Shield, Cambs, UK). Primary human monocytes were isolated by resuspending PBMCs in 5% human serum in RPMI and plating out in 12-well plates (10^6 cells per well) for 3 h to allow monocytes to adhere. Non-adherent cells were then washed off. The monocytes were allowed to differentiate into macrophages for 7–10 days.

For the phagocytosis assay, IMR-32 were labelled with the red fluorescent membrane dye PKH26, according to the manufacturer's instructions, and allowed to adhere overnight. Apoptosis was induced in IMR-32 using CPT or UV as described. In some experiments, established apoptotic cells (floating or loosely adherent) were separated from adherent surviving and early apoptotic cells by striking the plate and separately harvesting the two populations. Macrophages were washed twice in serum-free RPMI, labelled with 2 μM calcein. AM for 45 min, and then washed twice in medium containing 5% FCS. Labelled apoptotic or control IMR-32 cells (5×10^5) were plated onto the near-confluent adherent macrophage monolayer with C8d (20%) or HI-C8d (20%) in RPMI. After 2 h, remaining IMR-32 were washed from the macrophage monolayer with three washes of PBS. Coverslips were then fixed in acetone for 30 s, stained with DAPI, and mounted using Vectashield (Vector Laboratories, Peterborough, UK). Samples were analysed using a Leica fluorescence microscope (Leica Microsystems, Milton Keynes, UK) and a Compucyte laser scanning cytometer (LSC; Olympus, London, UK).

2.7. Statistics

Statistical analysis to compare differences between two groups was performed using the Student's *t*-test; where more than two groups were compared, one-way ANOVA with post hoc analysis was used. Results are expressed as mean ± S.E.M. or ± S.D., as indicated in the text.

3. Results

3.1. Apoptotic IMR-32 neuroblastoma cells lose CD46 and CD59

Apoptosis was induced in IMR32 using CPT, ceramide or UV irradiation. Fig. 1 shows that each protocol induced apoptosis in IMR32 as assessed using annexin-V-FITC and PI. Of note, the UV treated population demonstrated an annexin+/PI intermediate + population, likely representing early apoptotic cells as previously reported (Zamai et al., 1996). In each case CD46 levels were reduced by 80–90% on a subpopulation of cells that correlated with the observed percentage of apoptotic cells. CD59 levels were also altered on CPT and UV treated cells, but not ceramide treated cells. CPT treated cells gave two clear populations of CD59 expressing cells, one with reduced expression and one with increased expression compared to controls. The proportion showing reduction in CD59 expression correlated with the proportion of apoptotic cells in both CPT and UV treated cells; CD59 expression in this population was reduced by 25–30%.

We next performed time course studies on CPT treated IMR-32 (Fig. 2), as the stages of apoptosis were clearly defined and easy to follow. These studies showed that CD46 was lost early during apoptosis, and the proportion of cells losing CD46 mirrored the proportion of cells undergoing apoptosis. CD59 expression changed much later, and the two populations of cells observed previously were clearly defined only after 48 h. These experiments suggested that the loss of CD46 and CD59 was

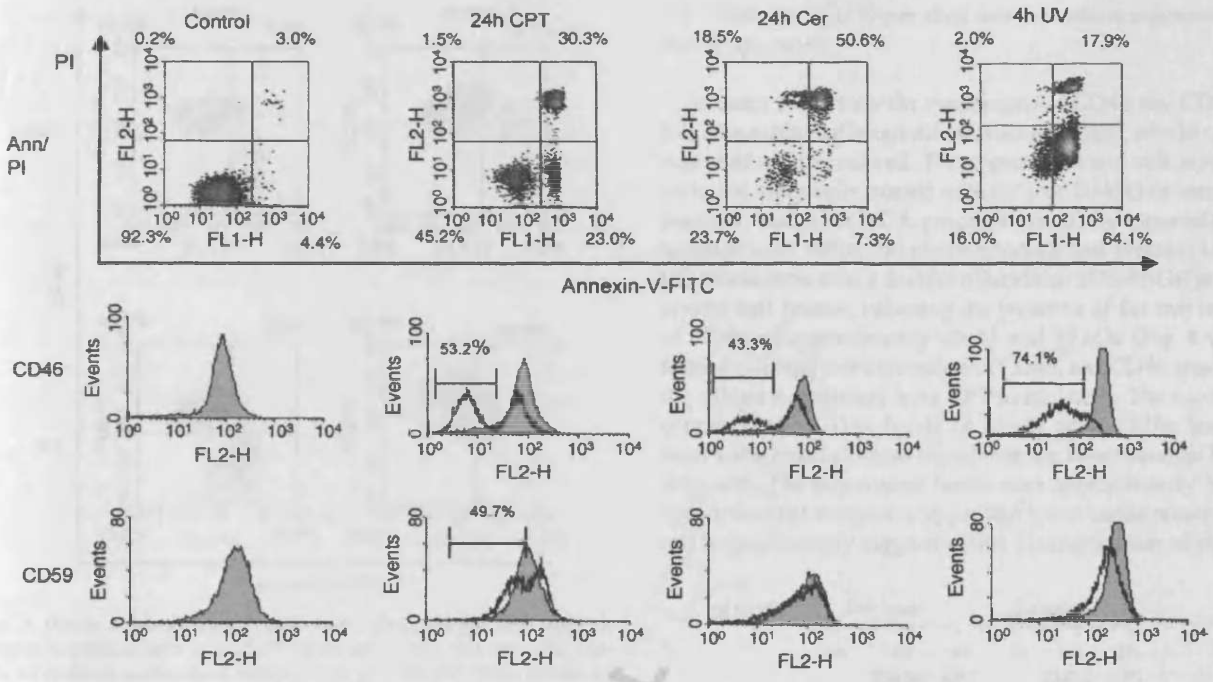

Fig. 1. Induction of apoptosis and CReg profiles in CPT treated, ceramide treated and UV irradiated IMR-32. Viable cells: annexin−/PI−. Early apoptotic cells: annexin+/PI−. Late apoptotic and necrotic cells: annexin+/PI+. All induction agents caused IMR-32 to lose CD46 and CD59. The numbers of cells in the low expressing population were similar to the numbers of apoptotic cells present (early + late apoptotic cells), particularly in relation to CD46. Filled profiles indicate the control populations at the indicated time point, and the black lines represent the treated populations. Markers indicate cells expressing low levels of CReg, with percentage of cells in this subpopulation specified above the marker. Results shown are representative of three to five separate experiments.

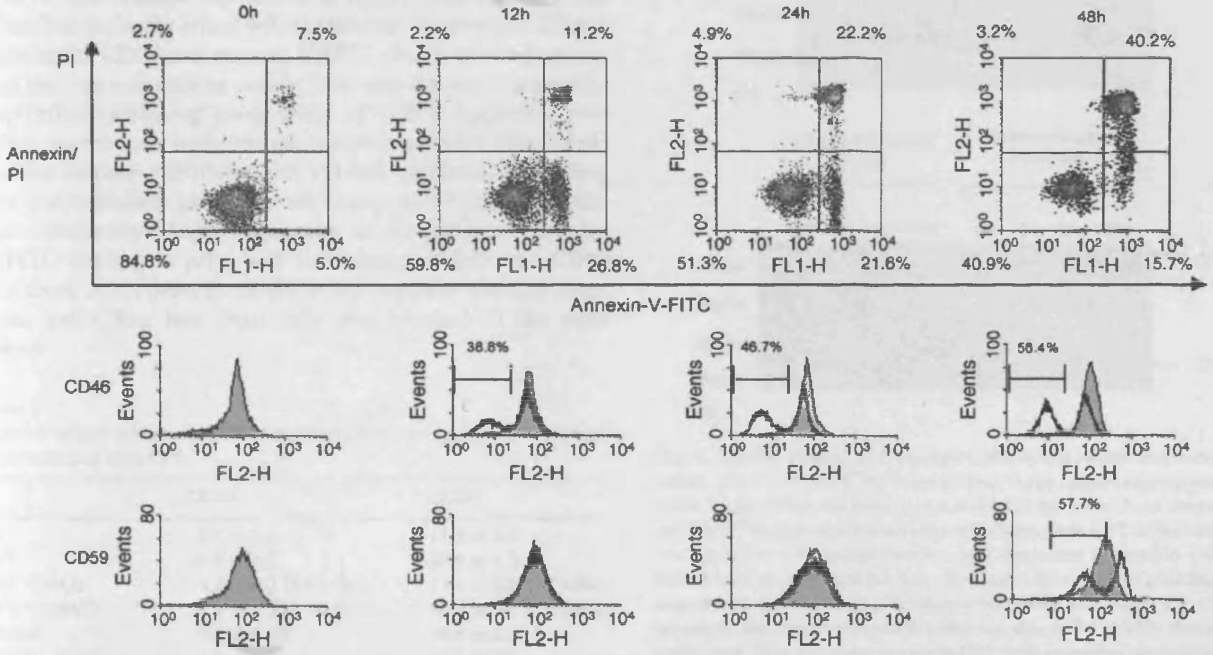

Fig. 2. Time course study of CD46 and CD59 loss from CPT treated cells. Loss of CD46 was evident as soon as cells became positive for early apoptotic markers, and the low expressing population increased as apoptosis progressed. CD59 loss was not evident early in apoptosis, but as apoptosis progressed high and low expressing populations of cells become apparent, and were clearly definable after 48 h of treatment. Filled profiles indicate the control populations and black lines represent the CPT treated populations. Markers represent the subpopulation of cells expressing low levels of CReg. Results are representative of three experiments.

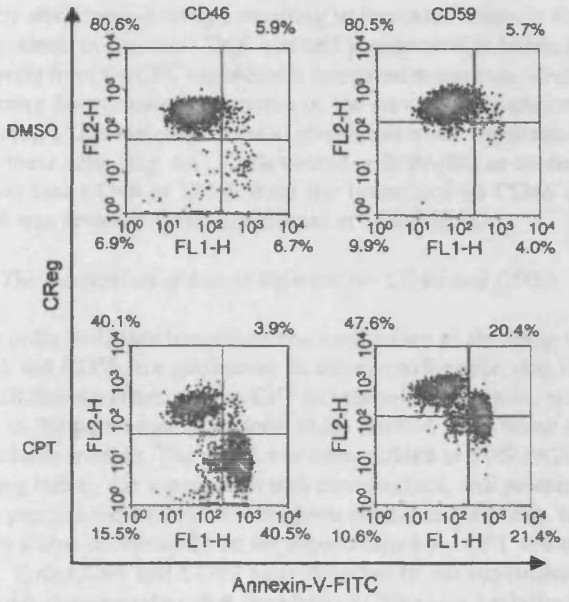

Fig. 3. Double staining apoptotic IMR-32 for CReg and apoptosis markers. Distinct populations after 24 h of CPT treatment confirm that only cells positive for apoptosis markers have reduced levels of CD46 and CD59. Results are representative of three experiments.

occurring on cells undergoing apoptosis, and that loss of CD46 was occurring earlier than loss of CD59.

In order to confirm that loss of CD46 and CD59 occurred on apoptotic cells, double staining for CReg with annexin-V-FITC was performed. Fig. 3 shows that only cells positive for annexin-V-FITC had reduced expression of CD46, demonstrating that CReg loss is closely linked with the process of apoptosis. Double staining for CD59 and annexin-V-FITC clearly showed separation into two populations even at 24 h, with the annexin positive population expressing lower levels of CD59. Apoptosis inhibition experiments using the pan-caspase inhibitor zVAD-fmk, and the caspase-3 inhibitor zDEVD-fmk confirmed that CReg loss was intimately associated with apoptosis (Table 1). zVAD-fmk completely blocked apoptosis as assessed by annexin-V-FITC binding to cells, and also blocked CD46 and CD59 loss from cells (Table 1). zDEVD-fmk partially blocked apoptosis, and CReg loss from cells was blocked to the same extent.

Table 1
Effect of caspase inhibition on CD46 and CD59 loss from apoptotic IMR32 after 24 h incubation with CPT

	CD46	CD59
0 h	8.7 ± 0.2	11.5 ± 0.2
CPT	46.0 ± 1.7	29.8 ± 1.3
CPT + zVAD	9.4 ± 0.3 ($p < 0.001$)	13.1 ± 0.6 ($p < 0.001$)
CPT + zDEVD	22.4 ± 0.6 ($p < 0.001$)	16.0 ± 1.5 ($p < 0.001$)
DMSO	7.5 ± 0.09	10.9 ± 0.3
DMSO + zVAD	5.9 ± 0.2	9.2 ± 0.4
DMSO + zDEVD	6.2 ± 0.1	9.6 ± 0.7

Results are expressed as percent of cells with low CReg expression on the cell surface + S.E.M. ($n = 3$).

3.2. CD46 and CD59 are shed into the culture supernatant during apoptosis

In order to identify the mechanism of CD46 and CD59 loss from the surface of apoptotic cells we examined whether protein was shed or internalised. The supernatant and cell layer were collected from cells treated with CPT or DMSO in serum-free medium, and, after TCA precipitation of the supernatant, the samples were subject to electrophoresis and Western blotting. CD46 was present as a doublet of bands on SDS-PAGE gels from control cell lysates, reflecting the presence of the two isoforms of CD46 of approximately Mr 55 and 59 kDa (Fig. 4A). CPT treated cells had lost expression of CD46, and CD46 appeared in the culture supernatant from CPT treated cells. The supernatant contained two CD46 bands of Mr 47 and 51 kDa; the upper band had a much stronger signal than the lower band on blotting (Fig. 4B). The supernatant bands were approximately 5–8 kDa lighter than the respective upper and lower bands present in the cell lysate, strongly suggesting that the mechanism of shedding

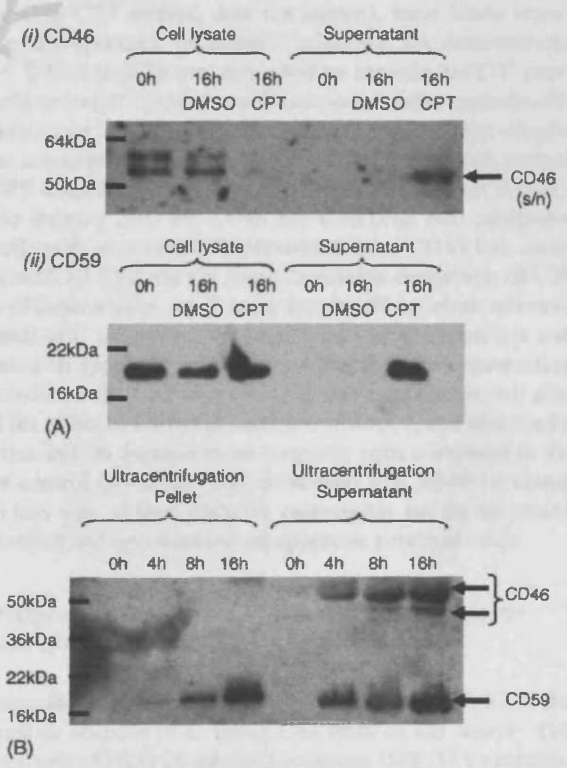

Fig. 4. Western blotting of CD46 and CD59 in cell lysates and culture supernatant. (A) CD59 and CD46 were present in the culture supernatant of CPT treated cells. CD46 was present as a doublet in the lysate from untreated (0 h) and DMSO treated cells, but not seen in the lysate from CPT treated cells. CD59 was present in CPT treated cells at a level equivalent to controls. Cell lysates loaded with 20 μg protein per lane. (B) Time course of CReg shedding into the supernatant and partitioning by ultracentrifugation at 100,000 × g. CD59 was present in the ultracentrifugation pellet and also in the soluble fraction of the supernatant from 4 h post-exposure to CPT, with increasing amounts present as the time of exposure increased. CD46 was present in the supernatant fraction in increasing amounts from 4 h, and a second lower Mr form (approx. 2 kDa lighter) was detectable from 8 h, and was absent from the UC pellet at all time points.

was by enzymatic cleavage, resulting in truncated forms in the supernatant. In contrast CD59 was still present at high levels in the lysate from the CPT treated cells compared to controls, likely reflecting the increased expression on the surviving population. However, CD59 was also found in abundance in the supernatant from these cells (Fig. 4A). Cells treated with DMSO as control did not lose CD46 or CD59 from the lysate and no CD46 or CD59 was detected in the supernatant at 16 h (Fig. 4A).

3.3. The mechanism of loss is different for CD46 and CD59

In order to further investigate the mechanism of shedding of CD46 and CD59, we performed an ultracentrifugation step on the cell-free supernatant from CPT treated cells to separate proteins in the particulate (apoptotic bleb) fraction from those in the soluble fraction. The pellet was resuspended in SDS-PAGE loading buffer; the supernatant was concentrated, and proteins TCA precipitated. Western blotting was performed, and Fig. 4B shows a time course study on the supernatant from CPT treated cells. Both CD46 and CD59 were detected in the supernatant from 4 h, demonstrating that shedding of CReg was a relatively early event. Both proteins accumulated in the supernatant to 16 h. CD46 was absent from the ultracentrifugation pellet, further supporting our suggestion that it was cleaved from the membrane of apoptotic cells. In contrast, CD59 was strongly detected in the ultracentrifugation pellet, present in increasing amounts from 4 h, indicating that it was shed on apoptotic blebs. Of note, CD59 present in the soluble fraction had a Mr 2–3 kDa lighter than that on apoptotic blebs, suggesting that it may also undergo a cleavage event to generate a truncated soluble form.

3.4. CD46 loss is MMP-dependent, while CD59 is loss is MMP independent

Since CD46 is shed as a soluble form and Western blotting data suggested a reduction in apparent Mr we hypothesised that CD46 was enzymatically cleaved from the cell surface during apoptosis, and that this was likely to be due to MMPs/ADAMs, since tumour cells have recently been shown to shed CReg by this mechanism (Hakulinen et al., 2004). To test this, we incubated CPT treated and control cells with or without the broad-spectrum MMP/ADAM inhibitor GM6001. The DMSO carrier (no. GM6001) inhibited CPT-induced apoptosis to a small degree (10% fewer apoptotic cells), and GM6001 had no additional effect on the progression of IMR-32 through apoptosis (Fig. 5A). Fig. 5B shows that CD46 loss from the CPT treated apoptotic cells was inhibited by GM6001 at 24 h; in contrast GM6001 had no effect on CD59 loss. This confirmed that an MMP/ADAM-mediated cleavage event was taking place for CD46, and demonstrated that CD59 loss occurred via an MMP independent mechanism. To further define which MMPs were involved in the cleavage of CD46, we tested specific inhibitors against MMP-2, MMP-3, MMP-8 and MMP-9, and also TIMP-3, an endogenous inhibitor of ADAMs. Fig. 5C shows that inhibitors of MMP-3 and MMP-8 were the most potent cleavage inhibitors; partial inhibition was also seen with MMP-9 inhibition. No effect was seen with MMP-2 inhibition or with the ADAM inhibitor TIMP-3. Combined inhibition of MMP-3 and MMP-8 blocked the majority of CD46 loss, indicating that these MMPs worked in combination. Combinations of various MMP inhibitors with MMP-9 inhibition resulted in no increase in effect above that noted for MMP-9 inhibition alone, suggesting that MMP-9 inhibition interfered with the effects of other inhibitors; we do not have an explanation for this phenomenon. Complete inhibition of CD46 loss was not achieved with any combination of specific inhibitor in these experiments, suggesting that other MMPs not examined in our study were also involved.

3.5. Apoptotic IMR-32 activate C more readily

As apoptotic IMR-32 lose CReg, we hypothesised that this would alter their susceptibility to C activation and opsonisation. We first assessed C1q binding and showed that control and apoptotic IMR-32 both bound C1q; the level of C1q binding was similar on the two populations (data not shown). A small population of PI+ cells had high C1q binding (12% controls versus 17% CPT treated, data not shown), most likely representing late apoptotic or necrotic cells. Fig. 6A demonstrates that 10-fold more C3b was deposited on annexin-V-FITC positive cells in the CPT treated population compared to control cells, indicating that apoptotic cells activated C more readily despite similar amounts of C1q binding. C3b deposition on both control and CPT treated cells was calcium dependent (data not shown). Double staining cells for CD46 and C3b (Fig. 6B) confirmed that cells with increased C3b deposition were CD46 low. Incubation with HI-C8d did not cause detectable deposition of C3b in any of these studies, confirming that the effects observed were the result of C activation. We confirmed that C deposition was increased on apoptotic cells by repeating the experiment using UV irradiated IMR-32 (Fig. 6C). In this experiment, we also tested the effect of the MMP inhibitor GM6001, and observed a reduction in C3b deposition on apoptotic cells compared to the carrier control ($p < 0.05$). This confirmed that MMP-mediated CD46 loss was, at least partially, responsible for the increased C activation and opsonisation on apoptotic neuronal cells.

3.6. Phagocytosis of apoptotic IMR-32 is enhanced in the presence of C

Macrophages were fed fluorescence labelled IMR-32 in the presence or absence of C, using C8d NHS as the source. The phagocytosis of PKH-26-labelled apoptotic IMR-32 by calcein-labelled macrophages was apparent by fluorescence microscopy (Fig. 7A). LSC analysis showed that uptake of non-apoptotic, non-opsonised IMR-32 was inefficient (3.9% positive, Fig. 7B) and opsonisation increased phagocytosis (9.0% positive). Apoptotic cells were more efficiently phagocytosed and this was further enhanced by opsonisation (18% positive). These data were analysed statistically in multiple experiments to compare uptake of opsonised and non-opsonised cells (Fig. 7C). There was a highly significant increase in phagocytosis following opsonisation of cells induced to apoptosis by CPT treatment or UV irradiation.

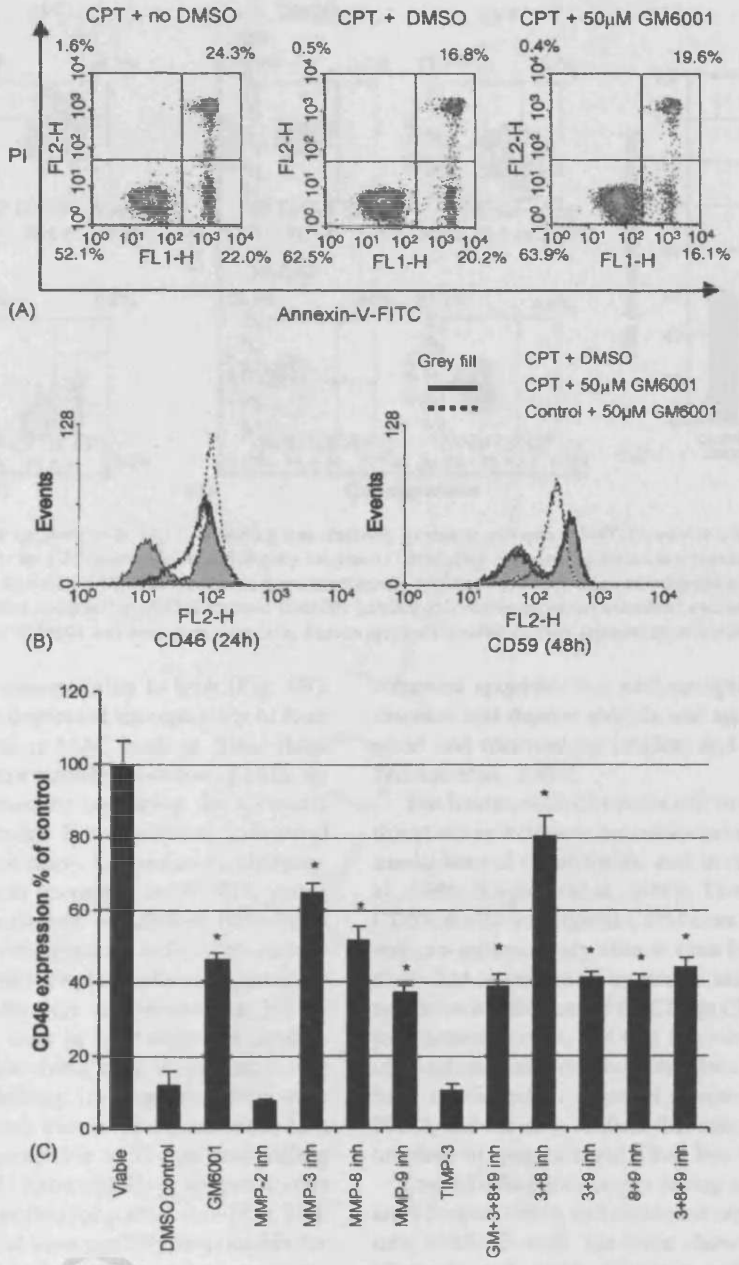

Fig. 5. Effect of MMP inhibition on CD46 and CD59 loss from apoptotic IMR-32. The MMP inhibitor GM6001 (50 μM) was added to CPT treated and control cells during apoptosis induction. (A) Apoptosis analysis using annexin-V-FITC/PI showed that DMSO (carrier control for GM6001) caused a small inhibition of CPT-induced apoptosis, but GM6001 had no additional effect at the concentrations used. (B) CD46 loss was inhibited by GM6001, but there was no effect on CD59 loss even after 48 h. Results are representative of three experiments. (C) MMP-3 and MMP-8 inhibitors partially blocked loss of CD46 and showed a greater effect when combined. MMP-9 inhibitor also partially blocked CD46 shedding. Results expressed as percentage of CD46 expression in control. Viable cells set at 100%, and all others were CPT treated with either carrier (DMSO control) or MMP inhibitors. All MMP inhibitors were used at 50 μM. Significant differences are in comparison with DMSO control; $^{*}p < 0.001$.

3.7. Early apoptotic IMR-32 are more susceptible to C-mediated lysis

We next examined the effect of apoptosis on binding of MAC and susceptibility to C-mediated lysis, as the altered expression of CD59 may affect this process. CPT treated and control cells were incubated with NHS or HI-NHS in CFD for 1 h. Cell death was assessed by measurement of LDH in the cell-free supernatant, and MAC deposition was assessed by staining with an anti-C9 neoepitope mAb (B7) and analysis by flow cytometry. Despite the large increase in C activation observed on apoptotic cells (Fig. 6A), after 12 h of CPT treatment there was no change

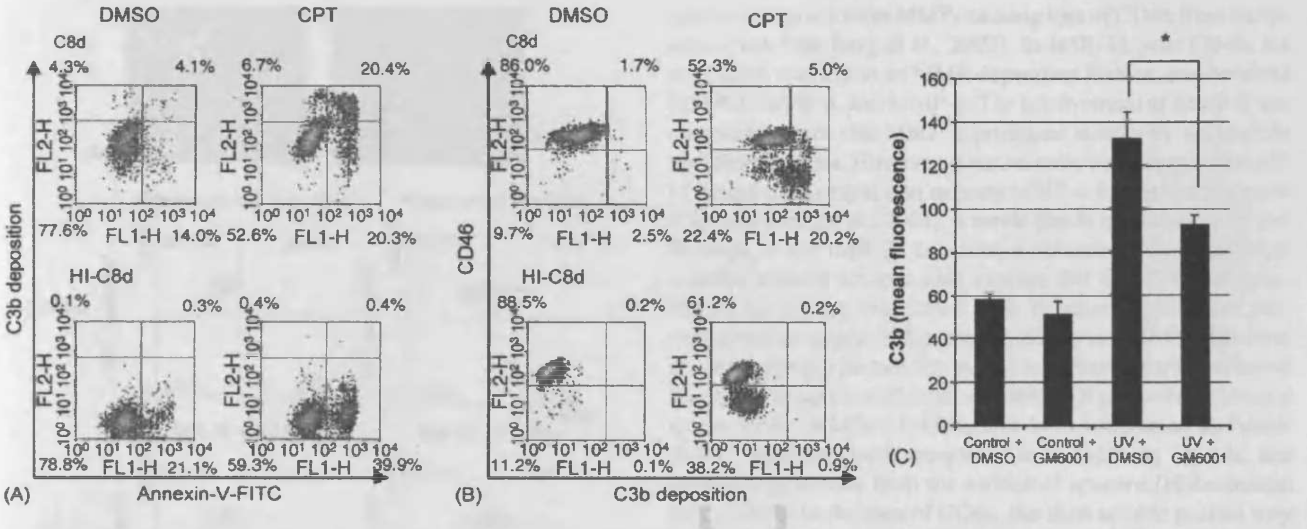

Fig. 6. C activation and deposition on apoptotic cells. (A) C3b binding was markedly increased on annexin-V-FITC positive cells following CPT treatment. The annexin-V-FITC negative population in the CPT treated group had slightly increased C3b binding compared to the control population. (B) Deposition of C3b was highest on cells that had lost most of their CD46. HI-C8d controls in these experiments confirmed that deposition was dependent on C activation. (C) UV-treated IMR-32 showed increased C3b deposition compared to DMSO controls. GM6001 (present only during apoptosis induction) attenuated the increased C activation on apoptotic cells ($^{*}p < 0.01$). No effect of GM6001 was seen on control cells. Results are representative of three separate experiments.

in MAC binding (Fig. 8A) or susceptibility to lysis (Fig. 8B). After 24 h of CPT treatment, a decreased susceptibility to lysis was evident, despite an increase in MAC binding. Since these experiments were carried out on a mixed population of cells, we further analysed this phenomenon by separating the apoptotic cells (floating or loosely adherent) from surviving (adherent) cells and subjected them to a lysis assay. Separation yielded populations with 90–95% of cells in apoptosis, or 90–95% viable cells (data not shown). At 12 h the two populations behaved in a markedly different manner, with apoptotic cells more susceptible to C-mediated killing, and surviving cells more resistant (Fig. 8C). At 24 h, apoptotic cells were more resistant to killing than controls, suggesting that cells in later stages of apoptosis were more resistant to C; surviving cells at 24 h were also more resistant to C-mediated killing. UV irradiated cells were then subjected to lysis assays, and, like apoptotic cells after 12 h CPT treatment, were more susceptible to C-mediated killing than controls (Fig. 8D). GM6001 reduced lysis of apoptotic cells ($p < 0.05$), but this was still higher than for control cells (Fig. 8D), indicating that CD46 loss was at least partially responsible for the increased killing. GM6001 had no effect on lytic susceptibility of non-apoptotic cells.

4. Discussion

The purpose of apoptosis is to enable the elimination of damaged, infected or otherwise compromised cells in a manner that does not cause inflammation (deCathelineau and Henson, 2003; Elward and Gasque, 2003). When a cell dies by apoptosis it is therefore important that it is efficiently recognised and removed by phagocytes before secondary necrosis occurs with consequent release of pro-inflammatory mediators. This process is particularly important in the nervous system where neurons are exquisitely sensitive to many of the mediators of inflammation. Neuronal apoptosis is a well-recognised feature of many brain diseases and disease models and apoptotic neurons are recognised and removed by resident and recruited phagocytes (De Simone et al., 2004).

The human neuroblastoma cell line IMR-32 was selected for this study as it closely resembles primary human neurons in the mechanism of C activation, and in its CReg profile (Gasque et al., 1996; Singhrao et al., 2000). These cells express CD46 and CD59, but do not express CD55 even after stimulation with several pro-inflammatory stimuli (van Beek et al., 2005). We here show that induction of apoptosis using physical and chemical agents causes the loss of the CRegs CD46 and CD59 from apoptotic neuronal cells, and that this can be blocked by inhibition of apoptosis using caspase inhibitors. Caspase-3 has previously been implicated in neuronal apoptosis (Yakovlev and Faden, 2001), and our data confirm that caspase-3 is the major caspase involved in apoptosis and CReg loss in our model.

Loss of CReg also occurs during neutrophil apoptosis (Jones and Morgan, 1995), and decreased expression of CD59 on apoptotic CD8+ T cells has been shown in autoimmune disease (Tsunoda et al., 2000). However, neither of these studies investigated the mechanism of CReg loss. We here demonstrate that CD46 was shed in a truncated soluble form, and that CD59 was lost on apoptotic blebs, and also as a soluble form. Interestingly, we detected both soluble and bleb-associated CD59 shedding by Western blot much earlier than CD59 loss from apoptotic cells was apparent by flow cytometry. Active shedding of CD59 therefore appears to occur early but continued synthesis maintains CD59 expression until late in the apoptotic process when energy stores are depleted and the cell loses membrane integrity. Others have shown that CD59 is shed on vesicles following CPT treatment of endothelial cells, even at doses insufficient to induce detectable apoptosis, supporting our assertion that this is an early event in apoptosis (Simak et al., 2002). We have previously

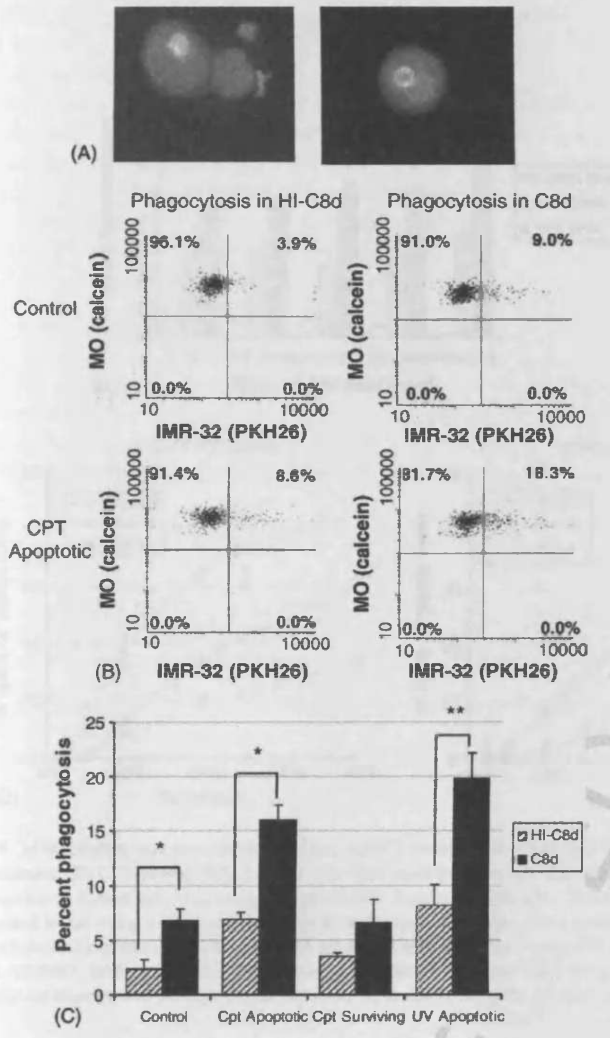

Fig. 7. Phagocytosis of apoptotic IMR-32 in the presence and absence of C. (A) Macrophages phagocytose apoptotic IMR-32. Macrophages were labelled with calcein (green), and IMR-32 were labelled with PKH26 (red). Apoptotic cells can be observed within the macrophages. Magnification ×40. (B) Laser scanning cytometry (LSC) of phagocytosis. Events in the upper right quadrant represent macrophages that have phagocytosed one or more IMR-32. (C) Phagocytosis of control and apoptotic IMR-32. Apoptotic IMR-32 were more readily phagocytosed than controls and cells surviving CPT treatment, and this was further enhanced by the presence of C. $^{*}p < 0.01$, $^{**}p < 0.001$. Results are representative of three experiments.

shown that CD59 and CD55 are present on exosomes, microparticles shed by T cells and involved in antigen presentation, and protect against C-mediated lysis (Clayton et al., 2003). Events placing GPI anchored molecules on vesicles may all occur via a common mechanism, and CD59 may have an important and common function in protecting apoptotic blebs, exosomes, and other shed particles from C attack.

The mechanism of shedding soluble CD46 and CD59 was investigated using MMP inhibitors, as others have shown that these enzymes are responsible for shedding of CReg from tumour cells (Hakulinen et al., 2004). Indeed, MMP-mediated CD46 shedding is also reported in other contexts: Loxoceles spider venom activates MMPs causing loss of CD46 from leukocytes (Van Den Berg et al., 2002). In IMR-32 cells CD46, but not CD59, was lost in an MMP-dependent fashion, and involved MMP-3, MMP-8, and MMP-9. The involvement of MMP-8 was surprising, since this MMP is produced mainly by neutrophils and chondrocytes. However, it has recently been shown that cells of neural crest origin also express MMP-8 during development (Giambernardi et al., 2001), a result that is in keeping with our findings in the IMR-32 cell line; it remains to be established whether mature neurons also express this MMP. Importantly, MMPs have been implicated both in neurodegenerative processes and in apoptosis (Rosenberg, 2002), and MMP-3 has been shown to act as a stimulatory signal for microglia when released by apoptotic neurons (Kim et al., 2005). Of particular relevance to our study, MMPs/ADAMs have been implicated as "sheddases", cleaving death receptors, death-inducing ligands, and adhesion molecules from the surface of neurons (Hubschmann et al., 2005). In the case of CD46, the shed soluble protein may have a protective, anti-inflammatory role in the environment surrounding the apoptotic cells by controlling local C activation, perhaps even after the dying cell has been cleared. Indeed, CD46 cleaved from tumour cells by MMPs retained its cofactor activity (Hakulinen et al., 2004).

We examined the route of C activation on neuronal cells, and found that C1q bound to both control and early apoptotic cells to the same degree; necrotic and late apoptotic cells bound more C1q than the rest of the population, in agreement with the observations of others (Gaipl et al., 2001; Zwart et al., 2004). Mannose binding lectin (MBL) has also been shown to bind to apoptotic cells and activate C, and like C1q this also occurs on late apoptotic cells (Nauta et al., 2003); we did not examine this in our study. We found that C activation on both control and apoptotic populations was calcium dependent, and this observation, coupled with the C1q binding data, suggested that the classical pathway was the route of C activation in our system. Although our data do not rule out a role for the lectin pathway, this route of activation has been shown to have a negligible role in opsonisation of apoptotic cells with C fragments in serum (Nauta et al., 2003).

A major functional consequence of opsonisation of cells by C is recognition and engulfment by phagocytes. We used primary human macrophages as a model of microglia, a relevant surrogate as these cell types share the same lineage (Gasque et al., 1995). CD46 loss resulted in increased opsonisation of apoptotic neuronal cells, and uptake of apoptotic cells by macrophages was increased in the presence of C. C-mediated promotion of clearance of apoptotic cells would limit inflammation, and this is in keeping with other published data describing enhanced phagocyte removal of C opsonised apoptotic targets (Mevorach et al., 1998; Takizawa et al., 1996). C1q has been reported to be the most physiologically relevant C opsonin for removal of apoptotic cells in most tissues in vivo (Taylor et al., 2000). However, in the context of inflammation, clearance of apoptotic cells may become increasingly dependent on C3b or other C opsonins to promote efficient disposal. Of note, inhibition of CD46 loss using MMP inhibitors markedly inhibited the enhancement of opsonisation of apoptotic cells, demonstrating conclusively that

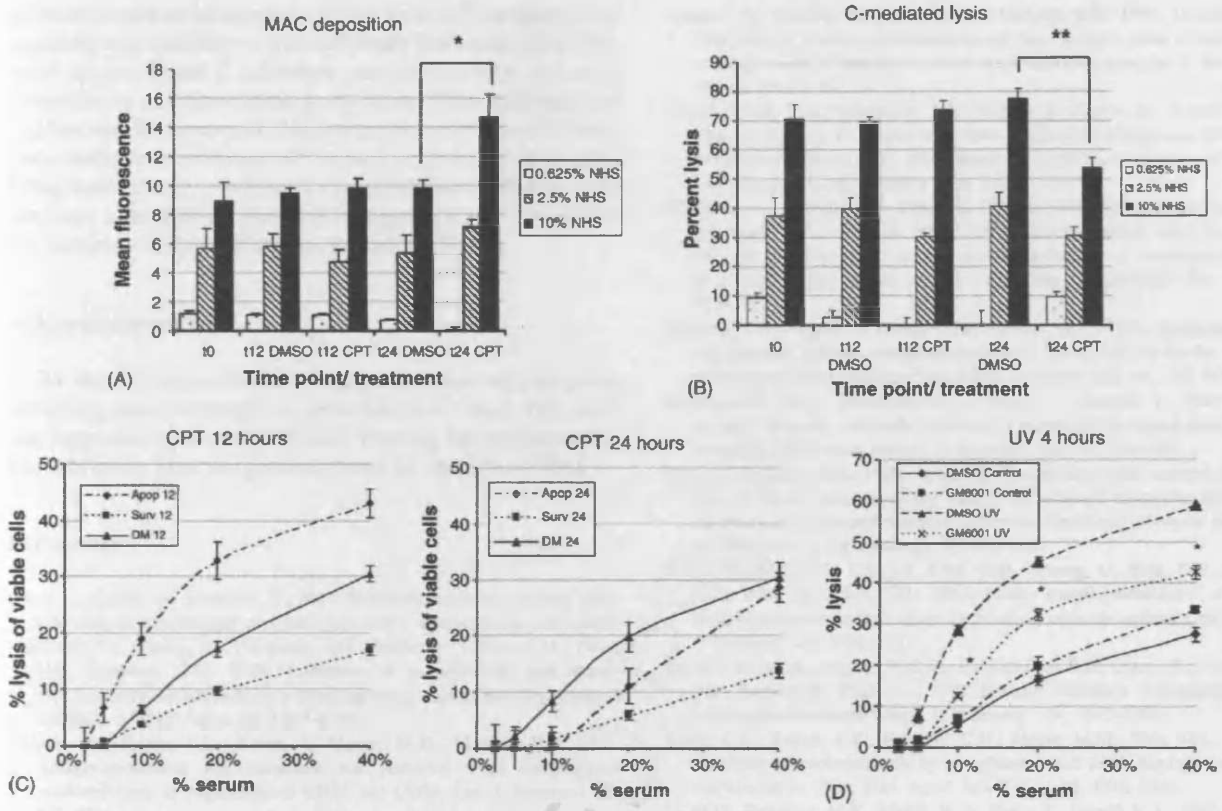

Fig. 8. MAC binding and susceptibility to lysis in CPT treated IMR-32. (A) CPT treated IMR-32 showed increased binding of MAC by flow cytometry after 24 h of treatment. (B) CPT treated IMR-32 after 24 h were more resistant to C-mediated lysis as detected by LDH release. (C) Apoptotic cells after 12 h CPT were more susceptible to C-mediated lysis compared to control. Apoptotic cells after 24 h CPT were more resistant to C compared to control, but more susceptible to lysis compared to surviving cells. Surviving cells at both time points were more resistant to lysis compared to control. (D) UV-treated cells were more susceptible to C-mediated killing than controls. Cells were incubated with GM6001 during UV irradiation and subsequent incubation, and washed thoroughly before exposure to NHS; GM6001 inhibited the increased C-mediated lysis on apoptotic IMR-32 ($p < 0.05$ compared with DMSO UV). No effect of GM6001 was seen on controls. Results are expressed as mean ± S.E.M. ($n = 4$ for A, B and D, $n = 6$ for C) from two or three combined experiments. $^{*}p < 0.05$, $^{**}p < 0.01$.

CD46 loss causes an increase in opsonisation. MMP inhibitors may be useful therapeutics in diseases where neuronal apoptosis and C activation occur. Indeed, MMP inhibitors have been successful in reducing damage in animal models of disease, including EAE (Clements et al., 1997; Hewson et al., 1995) and kainic acid-induced neuronal apoptosis (Campbell et al., 2004), although the relationship to C activation was not addressed in these studies. Our results reveal a novel mechanism by which these agents may act as neuroprotectants, and warrant further investigation.

Other anti-inflammatory responses to C activation were also noted in CPT treated cells. After 24 h, apoptotic and surviving cells were more resistant to C-mediated lysis than controls, despite more MAC binding. The resistance of surviving cells to C-mediated killing may be related to their higher levels of CD59 expression; however, apoptotic cells were also more resistant to lysis despite reduced expression of CD59. The data suggest that these cells express intrinsic lytic resistance mechanisms, independent of CD59. The phenomenon of CReg-independent resistance to C lysis has previously been reported, particularly in the context of accommodation in transplantation (Koch et al., 2005), and in the "induced protection" phenomenon where sublytic C attack primes cells to resist subsequent lytic C attack (Reiter et al., 1992). The observed increased resistance of late apoptotic cells to lytic killing suggests that neuronal cells induce protection mechanisms as apoptosis progresses, a finding that may be relevant in the context of more chronic insults to the brain.

Despite the anti-inflammatory responses noted above, early apoptotic cells were more susceptible to C-mediated lysis than non-apoptotic cells, in common with apoptosis in other cell types (Attali et al., 2004; Jones and Morgan, 1995). The potentially damaging consequence of increased lysis was reduced by the use of MMP inhibitors, clearly demonstrating that these compounds are neuroprotective under these circumstances in vitro. Importantly, early apoptotic cells were cleared efficiently by phagocytes following C opsonisation, indicating that disposal of neuronal apoptotic debris by resident phagocytes is likely to be critical in limiting damage during the acute phase of neuronal injury in vivo. Taken as a whole, our data suggest that apoptotic neuronal cells, stripped of the capacity to regulate C, activate C more readily. A delicate balance ensues between

efficient clearance of apoptotic debris (non-inflammatory) and increased susceptibility to lytic cell death (inflammatory). Neuronal apoptosis and C activation may therefore be important determinants of inflammation in the brain. These findings have implications for the use of C therapeutics in neurological disease, since therapeutic inhibition of C activation, either directly using CReg-based drugs, or indirectly via modulation of MMPs, could attenuate inflammation and limit damage, but may also impair the clearance of dying cells and exacerbate disease.

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References

Attali, G., Gancz, D., Fishelson, Z., 2004. Increased sensitivity of early apoptotic cells to complement-mediated lysis. Eur. J. Immunol. 34, 3236–3245.

Campbell, S.J., Finlay, M., Clements, J.M., Wells, G., Miller, K.M., Perry, V.H., Anthony, D.C., 2004. Reduction of excitotoxicity and associated leukocyte recruitment by a broad-spectrum matrix metalloproteinase inhibitor. J. Neurochem. 89, 1378–1386.

Clayton, A., Harris, C.L., Court, J., Mason, M.D., Morgan, B.P., 2003. Antigen-presenting cell exosomes are protected from complement-mediated lysis by expression of CD55 and CD59. Eur. J. Immunol. 33, 522–531.

Clements, J.M., Cossins, J.A., Wells, G.M., Corkill, D.J., Helfrich, K., Wood, L.M., Pigott, R., Stabler, G., Ward, G.A., Gearing, A.J., Miller, K.M., 1997. Matrix metalloproteinase expression during experimental autoimmune encephalomyelitis and effects of a combined matrix metalloproteinase and tumour necrosis factor-alpha inhibitor. J. Neuroimmunol. 74, 85–94.

Compston, D.A., Morgan, B.P., Campbell, A.K., Wilkins, P., Cole, G., Thomas, N.D., Jasani, B., 1989. Immunocytochemical localization of the terminal complement complex in multiple sclerosis. Neuropathol. Appl. Neurobiol. 15, 307–316.

Davie, C.A., Barker, G.J., Webb, S., Tofts, P.S., Thompson, A.J., Harding, A.E., McDonald, W.I., Miller, D.H., 1995. Persistent functional deficit in multiple sclerosis and autosomal dominant cerebellar ataxia is associated with axon loss. Brain 118 (Pt 6), 1583–1592.

De Simone, R., Ajmone-Cat, M.A., Minghetti, L., 2004. Atypical antiinflammatory activation of microglia induced by apoptotic neurons: possible role of phosphatidylserine–phosphatidylserine receptor interaction. Mol. Neurobiol. 29, 197–212.

deCathelineau, A.M., Henson, P.M., 2003. The final step in programmed cell death: phagocytes carry apoptotic cells to the grave. Essays Biochem. 39, 105–117.

Elward, K., Gasque, P., 2003. "Eat me" and "don't eat me" signals govern the innate immune response and tissue repair in the CNS: emphasis on the critical role of the complement system. Mol. Immunol. 40, 85–94.

Emmerling, M.R., Watson, M.D., Raby, C.A., Spiegel, K., 2000. The role of complement in Alzheimer's disease pathology. Biochim. Biophys. Acta. 1502, 158–171.

Gaipl, U.S., Kuenkele, S., Voll, R.E., Beyer, T.D., Kolowos, W., Heyder, P., Kalden, J.R., Herrmann, M., 2001. Complement binding is an early feature of necrotic and a rather late event during apoptotic cell death. Cell. Death. Differ. 8, 327–334.

Gasque, P., Fontaine, M., Morgan, B.P., 1995. Complement expression in human brain. Biosynthesis of terminal pathway components and regulators in human glial cells and cell lines. J. Immunol. 154, 4726–4733.

Gasque, P., Thomas, A., Fontaine, M., Morgan, B.P., 1996. Complement activation on human neuroblastoma cell lines in vitro: route of activation and expression of functional complement regulatory proteins. J. Neuroimmunol. 66, 29–40.

Giambernardi, T.A., Sakaguchi, A.Y., Gluhak, J., Pavlin, D., Troyer, D.A., Das, G., Rodeck, U., Klebe, R.J., 2001. Neutrophil collagenase (MMP-8) is expressed during early development in neural crest cells as well as in adult melanoma cells. Matrix Biol. 20, 577–587.

Hakulinen, J., Junnikkala, S., Sorsa, T., Meri, S., 2004. Complement inhibitor membrane cofactor protein (MCP; CD46) is constitutively shed from cancer cell membranes in vesicles and converted by a metalloproteinase to a functionally active soluble form. Eur. J. Immunol. 34, 2620–2629.

Hewson, A.K., Smith, T., Leonard, J.P., Cuzner, M.L., 1995. Suppression of experimental allergic encephalomyelitis in the Lewis rat by the matrix metalloproteinase inhibitor Ro31-9790. Inflamm. Res. 44, 345–349.

Hubschmann, M.V., Skladchikova, G., Bock, E., Berezin, V., 2005. Neural cell adhesion molecule function is regulated by metalloproteinase-mediated ectodomain release. J. Neurosci. Res. 80, 826–837.

Jones, J., Morgan, B.P., 1995. Apoptosis is associated with reduced expression of complement regulatory molecules, adhesion molecules and other receptors on polymorphonuclear leucocytes: functional relevance and role in inflammation. Immunology 86, 651–660.

Kim, Y.S., Kim, S.S., Cho, J.J., Choi, D.H., Hwang, O., Shin, D.H., Chun, H.S., Beal, M.F., Joh, T.H., 2005. Matrix metalloproteinase-3: a novel signaling proteinase from apoptotic neuronal cells that activates microglia. J. Neurosci. 25, 3701–3711.

Koch, C.A., Kanazawa, A., Nishitai, R., Knudsen, B.E., Ogata, K., Plummer, T.B., Butters, K., Platt, J.L., 2005. Intrinsic resistance of hepatocytes to complement-mediated injury. J. Immunol. 174, 7302–7309.

Koski, C.L., Ramm, L.E., Hammer, C.H., Mayer, M.M., Shin, M.L., 1983. Cytolysis of nucleated cells by complement: cell death displays multi-hit characteristics. Proc. Natl. Acad. Sci. U.S.A. 80, 3816–3820.

Li, M.O., Sarkisian, M.R., Mehal, W.Z., Rakic, P., Flavell, R.A., 2003. Phosphatidylserine receptor is required for clearance of apoptotic cells. Science 302, 1560–1563.

Mead, R.J., Singhrao, S.K., Neal, J.W., Lassmann, H., Morgan, B.P., 2002. The membrane attack complex of complement causes severe demyelination associated with acute axonal injury. J. Immunol. 168, 458–465.

Mevorach, D., Mascarenhas, J.O., Gershov, D., Elkon, K.B., 1998. Complement-dependent clearance of apoptotic cells by human macrophages. J. Exp. Med. 188, 2313–2320.

Meyer, R., Weissert, R., Diem, R., Storch, M.K., de Graaf, K.L., Kramer, B., Bahr, M., 2001. Acute neuronal apoptosis in a rat model of multiple sclerosis. J. Neurosci. 21, 6214–6220.

Morgan, B.P., 2000. Measurement of complement hemolytic activity, generation of complement-depleted sera, and production of hemolytic intermediates. Methods Mol. Biol. 150, 61–71.

Nauta, A.J., Raaschou-Jensen, N., Roos, A., Daha, M.R., Madsen, H.O., Borrias-Essers, M.C., Ryder, L.P., Koch, C., Garred, P., 2003. Mannose-binding lectin engagement with late apoptotic and necrotic cells. Eur. J. Immunol. 33, 2853–2863.

Reiter, Y., Ciobotariu, A., Fishelson, Z., 1992. Sublytic complement attack protects tumor cells from lytic doses of antibody and complement. Eur. J. Immunol. 22, 1207–1213.

Roederer, M., 2004. Conjugation of monoclonal antibodies. http://www.drmr.com/abcon/.

Rosenberg, G.A., 2002. Matrix metalloproteinases in neuroinflammation. Glia 39, 279–291.

Simak, J., Holada, K., Vostal, J.G., 2002. Release of annexin V-binding membrane microparticles from cultured human umbilical vein endothelial cells after treatment with camptothecin. BMC Cell. Biol. 3, 11.

Singhrao, S.K., Neal, J.W., Rushmere, N.K., Morgan, B.P., Gasque, P., 2000. Spontaneous classical pathway activation and deficiency of membrane regulators render human neurons susceptible to complement lysis. Am. J. Pathol. 157, 905–918.

Stolzing, A., Grune, T., 2004. Neuronal apoptotic bodies: phagocytosis and degradation by primary microglial cells. FASEB J. 18, 743–745.

Takizawa, F., Tsuji, S., Nagasawa, S., 1996. Enhancement of macrophage phagocytosis upon iC3b deposition on apoptotic cells. FEBS Lett. 397, 269–272.

Taylor, P.R., Carugati, A., Fadok, V.A., Cook, H.T., Andrews, M., Carroll, M.C., Savill, J.S., Henson, P.M., Botto, M., Walport, M.J., 2000. A hierarchical role for classical pathway complement proteins in the clearance of apoptotic cells in vivo. J. Exp. Med. 192, 359–366.

Tsunoda, S., Kawano, M., Koni, I., Kasahara, Y., Yachie, A., Miyawaki, T., Seki, H., 2000. Diminished expression of CD59 on activated CD8+ T cells undergoing apoptosis in systemic lupus erythematosus and Sjogren's syndrome. Scand. J. Immunol. 51, 293–299.

van Beek, J., van Meurs, M., t Hart, B.A., Brok, H.P., Neal, J.W., Chatagner, A., Harris, C.L., Omidvar, N., Morgan, B.P., Laman, J.D., Gasque, P., 2005. Decay-accelerating factor (CD55) is expressed by neurons in response to chronic but not acute autoimmune central nervous system inflammation associated with complement activation. J. Immunol. 174, 2353–2365.

Van Den Berg, C.W., De Andrade, R.M., Magnoli, F.C., Marchbank, K.J., Tambourgi, D.V., 2002. Loxosceles spider venom induces metalloproteinase mediated cleavage of MCP/CD46 and MHCI and induces protection against C-mediated lysis. Immunology 107, 102–110.

Yakovlev, A.G., Faden, A.I., 2001. Caspase-dependent apoptotic pathways in CNS injury. Mol. Neurobiol. 24, 131–144.

Zamai, L., Falcieri, E., Marhefka, G., Vitale, M., 1996. Supravital exposure to propidium iodide identifies apoptotic cells in the absence of nucleosomal DNA fragmentation. Cytometry. 23, 303–311.

Zwart, B., Ciurana, C., Rensink, I., Manoe, R., Hack, C.E., Aarden, L.A., 2004. Complement activation by apoptotic cells occurs predominantly via IgM and is limited to late apoptotic (secondary necrotic) cells. Autoimmunity 37, 95–102.

p53 regulates cellular resistance to complement lysis through enhanced expression of CD59

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Abstract

It has been recently hypothesized that the CD59 gene has two putative p53-responsive elements that may be involved in defence of host cells from damage by the complement system in inflammation. Here we have examined the roles of these putative p53-binding sequences within the CD59 gene in regulation of CD59 expression. We have demonstrated that both of these potential responsive elements bind p53 *in vitro*. Knocking down expression of p53 using siRNA led to a 6-fold decrease in CD59 protein expression in HeLa cells. We have previously observed a decrease of CD59 in camptothecin induced apoptotic IMR32 cells, while expression was increased in the surviving fraction compared to untreated cells. Here we have shown that these changes are associated with altered expression levels and acetylation status of p53. We have also demonstrated that acetylation status of p53 regulates CD59 expression on cells exposed to inflammatory cytokines to model inflammation. Our data suggest that p53, and *in vivo* positive/negative regulators of p53, could be used to modulate susceptibility of tumour cells to complement lysis in chemotherapy.

Introduction

Complement (C) is a major component of innate immunity. It eliminates invading microorganisms, transformed cells and molecular aggregates from tissues and biological fluids (1). Activation occurs through three convergent pathways, in which the C components are activated by sequential proteolytic cleavages and/or binding previously activated components. This results in the release of chemotactic factors and cell-activating anaphylatoxins, deposition of opsonic fragments and formation of the cytolytic membrane attack complex (MAC) (1). C may be activated on tumour cells by antibodies (2), immune complexes (3), as a consequence of apoptosis (4) or through proteolytic processes (5-7). However, the cytolytic activity of C is not always sufficient as an immunological surveillance mechanism, particularly against tumours (8, 9).

Both normal and malignant cells are protected by membrane-bound complement regulators (CReg) that either limit the formation of the C3/C5 convertase enzymes, or the assembly of the MAC (1, 8-10). The key C enzymes, C3/C5 convertases, are inhibited by the CReg C receptor 1 (CR1, CD35), decay-accelerating factor (DAF, CD55) and membrane cofactor protein (MCP, CD46). These molecules prevent assembly and promote decay of the C3/C5 convertases (CR1 and DAF), or serve as cofactors for the plasma serine protease factor I, which irreversibly inactivates C4b and C3b (CR1 and MCP). CD59, on the other hand, inhibits formation of MAC on cell surfaces. CReg are broadly expressed on a wide variety of tissues and cells, including epithelial, endothelial and circulatory cells, and act as physiologic brakes to C amplification. CD46, CD55 and CD59 are expressed on a number of solid tumours and associated cell lines and it is

apparent that the level of expression in malignant tissue is often greater than that seen in the normal surrounding tissue (7, 11-13). Consequently, the increased C resistance conferred by these membrane-bound CReg has been proposed as a mechanism that facilitates survival of the tumour or the metastasizing tumour cell when it enters circulation (8, 14, 15).

Little is known about the mechanisms by which expression levels of CReg are controlled in cells. P53, a broadly distributed tumour suppressor protein, is a regulator of expression of several proteins. P53 binds to double-stranded DNA in regions identical with or homologous to a consensus sequence containing at least two decamers of the type Pu Pu Pu C A/T T/A G Py Py Py, separated by 0 to 13 bp (16). The list of genes which possess these p53-binding sites is rapidly increasing and includes p21/WAF1, MDM2, GADD45, BAX, cyclin G, cyclin D, IGF-BP3, PCDNA, TGF-a, Ras and p53 itself (17-27). The p53-binding sites may be either in the promoter regions or within introns. P53 has recently been reported to enhance the transcription of a glycosyl-phosphatidylinositol (GPI)-linked membrane protein GML (28) that is related to apoptosis and participates in the sensitisation of malignant cells to chemotherapy and inflammation (29, 30). GML exhibits structural homology with the MAC inhibitor CD59 (31). Recently, potential p53-responsive elements have been identified in the CD59 gene by analyses *in silico* (32). A possible participation of p53 in the immune response by modulating the levels of CD59 has been hypothesized, based upon the observation that inflammation is associated with high expression levels of p53 (33, 34) and CD59 (35-37).

In the present study we have examined a potential involvement of the putative p53-binding sequences in the promoter and intron areas of the CD59 gene in regulation of CD59 expression. For the first time we have demonstrated a role for p53 and specifically acetylated p53 in modulation of CD59 expression in cells that may be important in inflammation and in immune escape of cancer cells.

Materials and Methods

Cells and treatments

Human epitheloid carcinoma (HeLa), hepatoma (Hep3B), neuroblastoma (IMR32) and promyelocytic leukaemia (HL60) cell lines were obtained from the European Collection of Animal Cell Cultures (ECACC; Salisbury, UK). HeLa and Hep3B were propagated in Dulbecco's modified Eagle's medium (DMEM) containing 10% heat-inactivated fetal calf serum (HI-FCS), supplemented with glutamine, penicillin and streptomycin (Invitrogen, Paisley, UK). IMR32 were maintained in RPMI 1640 with 5% HI-FCS and HL60 cells were grown in Iscove's modified Dulbecco's medium (IMDM) containing 20% HI-FCS, both supplemented as above.

Cytokines were purchased from PeproTech (London, UK) and were added to the cell medium of IMR32, Hep3B and HL60 for 36 hours at the following concentrations: IL-8, 25ng/ml, IFN-γ, 500U/ml.

To knock down expression of p53, HeLa cells were transfected using human specific p53 siRNA (Cell Signalling Technology, UK) following the supplier's protocol.

Induction of apoptosis

IMR32 (10^7 cells) were plated out in 175cm^2 flasks and allowed to grow for 24 to 48 hours until 80-90% confluent. Induction of apoptosis was achieved by replacing the medium with fresh RPMI 1640 with additives as above and 5% HI-FCS containing 1μM camptothecin (Sigma,Gillingham, Dorset, UK). Apoptotic cells were harvested at 24 or 48 hours post-induction by striking the flask gently 10 times and removing the medium containing apoptotic cells. Surviving IMR32 remained adherent, and these were harvested by brief trypsinisation. Analysis by flow cytometry using Annexin-V-FITC and PI showed 90-95% of loose and floating cells were apoptotic, and 5-10% in the adherent population were apoptotic (data not shown). Cells were then spun at 300g at 22°C for 3 minutes, and washed in serum-free RPMI before analysis.

Electrophoretic mobility shift assay (EMSA)

Oligonucleotides matching the two putative p53-binding sites within CD59 gene (CD59.1 and CD59.2) (Fig. 1*A*) and their complementary sequences were purchased from Invitrogen (Table 1). As a positive control we used sequence which is an exact match of a previously described p53-binding consensus sequence (20). For negative control we employed a sequence with complete mismatch to the p53 responsive element. Each oligonucleotide was incubated in PBS with its complementary sequence to obtain double-stranded DNA. All probes were labeled with horseradish peroxidase using the North2South Direct horseradish peroxidase labeling and detection kit (Perbio Science UK Ltd). Heat-denatured DNA probes (80ng each) were incubated with 100ng of wild-type

p53 protein (BD Biosciences, UK) for 50 min at 22°C in binding buffer (20 mM Tris-HCl pH 7.4, 0.1 mM EDTA, 0.1% Triton X-100, 1% 2-mercaptoethanol, 2 mM $MgCl_2$, 5% glycerol), containing 0.1 mg/ml poly-d(IC). Labeled probes alone and those from the incubation reactions were separated in a 2% agarose gel. The DNA signal was detected by chemiluminescence.

Western blot analysis

Cell lysates were subjected to SDS-PAGE on 10% gels. Proteins were transferred onto a nitrocellulose membrane and blocked using PBS/0.1% Tween20/5% non-fat dried milk for 1 hour at 22°C. Membranes were then incubated with primary antibodies overnight at 4°C in PBS/Tween20/milk (10ml), and then washed 3 times in PBS/Tween20 for 10 minutes on a rotamixer. Membranes were then incubated with the secondary antibody (donkey anti-mouse-HRPO, Biorad) for 1 hour at room temperature and washed 3 times in PBS /Tween. Membranes were developed using the ECL Western Blotting detection system (Pierce) and imaged using Kodak Medical Imaging film. Data were quantified densitometrically (Quantity One 4.3.0 software, BioRad) and triplicate measurements were evaluated statistically using Student's t-test. Primary antibodies used were: mouse monoclonal BRIC229 (International Blood Group Reference Laboratory, Bristol, UK) for detection of CD59; rabbit polyclonal anti-acetyl-p53(Lys373, Lys382) and mouse monoclonal anti-p53, clone BP53-12 (Upstate, UK) for detection of acetylated and total p53 respectively; rabbit polyclonal anti-p42 MAPK (Cell Signaling Technology) for p42 MAPK detection. We used either sheep anti-mouse-HRPO or sheep anti-rabbit-HRPO as secondary antibodies, both purchased from The Binding Site (UK).

Flow cytometry

Cells (4 x 10^5) were seeded into 12-well plates and, following incubation with cytokines (Hep3B and HL60), or transfection with the p53 siRNA (HeLa), were washed twice with PBS, then disaggregated with flow cytometry buffer (FCB; PBS containing 15mM EDTA, 1% bovine serum albumin (BSA), 15mM NaN_3, pH 7.4). For intracellular staining cells were permeabilized using BD CytofixCytoperm™ Kit (BD Biosciences). Cells were then resuspended at a concentration of 10^6/ml. Cells (100 µl; 10^5) were incubated with 5µg of specific primary antibody: BRIC229, anti-p53, clone BP-53-12, or anti-acetyl-p53 (Lys373, Lys382) for 30 min on ice, and the unbound antibody removed by three washes with FCB. The cells were then incubated with 1:100 dilution of the FITC-conjugated secondary antibody (anti-mouse or anti-rabbit immunoglobulins, The Binding Site), washed three more times with FCB and analysed on a BD FACScalibur (BD, Oxford, UK). All measurements were made in duplicate and each experiment was replicated twice. All results were combined and statistically analysed by Student's t-test. $P<0.05$ was considered to show statistically significant differences.

Complement lysis assay

Normal Human Serum (NHS), obtained by cubital vein puncture from healthy voluntaries, separated promptly and stored at –80°C until use, was the source of C in all experiments. As a negative control for the C-mediated lysis we either inactivated C by heat-treatment (15 min at 56°C) of NHS (HI-NHS) or depleted the NHS of C8 (C8d-NHS) using a monoclonal affinity column. Apoptotic and surviving IMR32, following camptothecin treatment, were harvested as described above, and washed twice in C

fixation diluent (CFD; Oxoid Basingstoke, UK). NHS or C8d-NHS were diluted as appropriate in CFD and added to 10^5 cells (final volume 100µl) and incubated for 1 hour at 37°C, 5% CO_2. Following incubation, samples were centrifuged at 300g for 3 minutes, the supernatant removed, and cells stained with 2µg/ml propidium iodide in FCB and analysed on the FL-2 channel of a BD FACScalibur flow cytometer. In some experiments untreated and surviving IMR32 cells were preincubated with the CD59 blocking antibody BRIC229 (20µg/ml) for 1h at 37 °C, washed twice with PBS and then the serum (NHS or C8d-NHS) was added at appropriate dilutions. Percent of lysed cells was calculated by the following equation:

% lysis ={(% lysis [cells/NHS] - % lysis [cells/C8d-NHS])/(100-% lysis[cells/C8d-NHS])}x 100

Chromatin Immuno-Precipitation (ChIP)

IMR32 cells treated with camptothecin for 24 or 48h were separated into apoptotic and surviving fractions, each fixed for 10 min at room temperature in tissue culture medium containing 1% formaldehyde. Untreated IMR32 cells were processed according to the same protocol as a control. All further steps of this assay have been described previously (38). Chromatin sonication was performed to produce DNA fragments in the range of 300–1000bp (electrophoretically determined in 1.5% agarose). The immunoprecipitation was performed with anti-acetyl-p53 (Lys373, Lys382) antibody (Upstate). The naked co-immunoprecipitated DNAs were then used as templates (10ng of DNA/reaction) in quantitative PCR assays (39) for detection of p53-responsive elements. A primer pair (negative/ChIP) designed to a sequence within the CD59 gene that does not bind p53 was

used as a control for the specificity of immunoprecipitation (Table 2). The same assay was carried out for Hep3B cells treated with either IL-8 or IFN-γ for 36h. Statistical significance of the data was assessed by the Student's t-test.

Quantitative RT-PCR (QPCR)

Total RNA was purified either from IMR32, IMR32 treated with cytokines (IL-8 or IFN-γ) or camptothecin, Hep3B and HL60, or Hep3B and HL60 treated with cytokines as with IMR32, using the RNeasy kit (Qiagen, Sussex, UK). Aliquots of these RNAs, 1 μg each, were reverse-transcribed using random hexamers and multiscribe reverse transcriptase according to the manufacturer's instructions (Applied Biosystems, Warrington, UK). Primer pairs were designed using Primer Express software (Applied Biosystems) to monitor expression of β-actin, p53, and CD59 genes (Table 2). Quantitative PCR was performed using TaqMan Universal PCR Master Mix according to the manufacturer's instructions (Applied Biosystems), with the exception that 25 μl reaction volumes were used, with 45 cycles of amplification. The concentrations of each of the primer pairs were optimised to ensure amplification of the specific product and the absence of primer/probe dimers. PCR was performed on the ABI PRISM 7000 (Applied Biosystems). The real-time PCR results were analyzed using the sequence detection system software version 1.9 (Applied Biosystems). RNA expression levels were calculated using the comparative Ct method (ΔΔCt) as described by manufacturer. ΔΔCt validation experiments showed similar amplification efficiencies for all templates used (difference between line slopes for all templates <0.1). At least two independent experiments were performed for each

mRNA and Student's t-test was applied to calculate significance in changes of expression pattern.

Results

p53 binds response elements in the CD59 gene and modulates its expression

Firstly, we investigated whether the two putative p53-responsive elements mapped in the CD59 gene (Fig. 1*A*) were capable of binding p53 protein *in vitro*. We performed EMSA for these two elements with recombinant wild type p53. As a positive control we used an oligonucleotide which exactly matches previously described p53-binding consensus sequence (20). For negative control we employed a sequence with complete mismatch to the p53 responsive element. These experiments (Fig. 1*B*) unambiguously demonstrated that both putative p53-binding sites within the CD59 gene were capable of interacting with p53 *in vitro*. To address further whether p53 plays a role in regulation of CD59 expression in living cells, we transfected HeLa cells with small interfering RNA (siRNA), which specifically causes degradation of p53 mRNA. We chose this cell line because we found it to express high levels of both p53 and CD59 (Fig. 2) and the p53 siRNA transfection kit we used for this experiment was optimised for HeLa. Thirty-six hours after the transfection we prepared cellular lysate which we used for Western analysis (Fig. 2*A*). Densitometric analysis demonstrated that expression of p53 and CD59 was reduced by 5 (SD±0.2)- and 6.5 (SD±0.3) - fold respectively. As a control for the siRNA transfection we carried out detection for p42 MAPK, which is known to be involved in degradation of p53 (40). We did not detect any change in expression of this protein suggesting that decreased expression of p53 is a result of the p53siRNA

transfection. Decreased expression of CD59 on HeLa cells transfected with p53 siRNA was confirmed by flow cytometry (Fig. 2*B*). Expression 36h after the transfection was reduced 6 (SD±0.2) - fold compared to untranfected cells.

Acetylation of p53 modulates CD59 expression in tumour cells exposed to camptothecin

Analogues of the apoptosis-inducing chemotherapeutic agent camptothecin have been used as second-line therapy in cancer patients (41, 42), with improved survival time compared to standard therapy. We have shown that treatment of the neuroblastoma cell line IMR32 with camptothecin induces loss of CD59 on the apoptotic cells (43). In order to investigate whether p53 was involved in these changes we first treated IMR32 with camptothecin and examined CD59 expression by Western blotting as above. We confirmed the reduced expression of CD59 in the apoptotic fractions by comparison with untreated cells 1.8 (SD±0.2) - fold at 24h and 2.3 (SD±0.2) –fold at 48h (Fig. 3*A*); however, in the surviving cells we detected a 3.6 (SD±0.3) - fold increase of CD59 expression at both 24 and 48h in comparison to controls. To confirm that gene regulation was responsible for the increased CD59 protein expression in surviving camptothecin treated IMR32 cells, we measured CD59 mRNA levels. In surviving cells CD59 mRNA was increased around 4-fold compared to untreated or treated apoptotic cells (Fig. 3*B*). Further, we examined whether the changes in CD59 expression correlated with susceptibility to C lysis (Fig. 3*C*). IMR32 spontaneously activated C without need for sensitising antibody as previously described (44). Cells surviving camptothecin treatment were more resistant to C, lysis being reduced to half that in untreated cells at the same C

dose. To confirm that CD59 was responsible for this effect we measured the lysis of IMR32 untreated and surviving cells, in which CD59 was first blocked by BRIC229 antibody. No difference in percent of lysed cells was observed in this experiment confirming that increased resistance of surviving cells to C lysis was a result of increased CD59 expression. Neither HI-NHS nor C8d-NHS caused specific lysis of IMR32 (data not shown).

In an attempt to elucidate the mechanisms modulating CD59 expression we investigated whether expression of p53 was influenced by camptothecin. Employing the IMR32 apoptotic model, we found that camptothecin treatment increased p53 expression in both apoptotic (2.4 SD±0.1-fold) and surviving (6.7 SD±0.2-fold) fractions (Fig. 4*A*). Moreover, when we used an antibody specific for the acetylated (transcriptionally active) form of p53, we detected a 5 (SD±0.3) - fold decrease in acetylated p53 in apoptotic cells and a 5.7 (SD±0.3) - fold higher acetylation in surviving cells compared to untreated IMR32 (Fig. 4*A*). These data suggest that changes in p53 levels and acetylation modulate CD59 expression in these cells.

To further investigate in camptothecin treated cells the role acetylation of p53 plays in upregulation of CD59 we carried out ChIP using anti-acetyl-p53 (Lys 373, Lys 382) antibody (Fig. 4*B*). Data showed that the acetylated form of p53 recognised by this antibody is recruited to both p53-responsive elements in the CD59 gene in surviving cells. However, in apoptotic fractions we detected around 3-fold less acetylated p53 molecules bound to these responsive sequences. These data confirm that acetylated p53

plays a key role in the regulation of CD59 expression in tumour cells (Fig. 4). Performing QPCR on immunoprecipitated DNA with a primer pair designed for a sequence within CD59 gene that does not bind p53 (negative/ChIP, Table 2) gave insignificant number of copies compared to that in genomic DNA in the same reaction (1460-fold less, data not shown), confirming very low background in our ChIP experiments.

Increased expression of CD59 in inflammation is dependent on acetylation of p53

It has been hypothesized recently that p53 is involved in the innate response to inflammatory stimuli by upregulating the expression of CD59 and thus protecting host cells from innate C lysis (32). To investigate this hypothesis and address possible mechanism we first treated IMR32 cells with cytokines specific for acute (IL-8) and chronic (IFN-γ) inflammation (45). However, QPCR data (not shown) did not demonstrate any effect of these cytokines on IMR32 expression of p53 and CD59. Therefore, we performed further investigations on human hepatoma cell line Hep3B, previously demonstrated to respond to IFN-γ by decreased CD59 expression (46). We carried out similar cytokine treatments as for IMR32 (Fig. 5). Expression of p53 mRNA was increased by 16% with IL-8 treatment and 26% with IFN-γ (Fig. 5*A*). However, CD59 mRNA expression was upregulated by IL-8 (26%) but decreased by IFN-γ (76% of expression in untreated Hep3B cells). To address this discrepancy between mRNA expression levels of p53 and CD59 upon cytokine treatment, we studied their protein levels (Fig. 5*B*). Expression of CD59 protein was markedly decreased in IFN-γ treated cells (40% of expression in untreated cells) but increased in IL-8 treated cells by 20% compared to untreated cells (Fig. 5*B*) correlating with the mRNA data. The overall

protein expression of p53 did not change with the cytokine treatments. However, when we used antibody specifically detecting (Lys373, Lys382) acetylated p53, we found a significant decrease (14±3.4%) in p53 acetylation when cells were treated with IFN-γ and increase (18±3.1%) upon IL-8 treatment. Changes in the acetylation of p53 therefore correlated with expression level of CD59 at mRNA and protein levels (Fig. 5). To address whether these small changes in acetylation of p53 were sufficient to account for the large decrease in CD59 protein expression, we carried out similar cytokine treatment of the p53 null HL60 cells (47). We did not detect significant change in CD59 mRNA upon IFN-γ and IL-8 treatment supporting the essential role for acetylated p53 (Fig. 5*A*). However, there was a slight but statistically significant decrease (12%) in expression of CD59 protein in HL60 cells incubated with IFN-γ (Fig. 5*B*). These data suggest that although changes in expression of CD59 mRNA upon IFN-γ treatment are related to the p53 changes only, this cytokine affects translational machinery as well. The effect of inhibition of protein synthesis by IFN-γ has been demonstrated previously (48, 49). Therefore, changes in p53 acetylation pattern and inhibition of mRNA translation result in synergistical downregulation of expression of CD59 protein upon treatment with IFN-γ.

To confirm that changes in the acetylation pattern of p53 upon treatment of Hep3B cells affect binding of p53 to its responsive elements in the CD59 gene, we performed ChIP assay using antibody that is specific for (Lys373, Lys382) acetylated p53 (Fig. 6*A*). Our data showed that acetylated p53 was recruited to both binding sites within the CD59 gene in Hep3B cells treated with IL-8. This recruitment correlated with the increased

acetylation of p53 (Fig. 5*B*). However, when cells were incubated with IFN-γ, acetylation of p53 was decreased (Fig. 5*B*) and the p53-responsive elements in CD59 gene bound less p53 (Fig. 6*A*). To address whether the decreased binding of p53 to the responsive elements in the CD59 gene is due to reduced availability of acetylated p53 molecules, we carried out another ChIP with the antibody specific for all forms of p53 (Fig. 6*B*). Although overall expression of p53 protein did not change upon cytokine treatments (Fig. 5*B*), total p53 ChIP closely mirrored that of acetylated p53 (Fig. 6*A, B*), confirming that acetylation is necessary for binding of p53 to its responsive elements in CD59 gene.

To control specificity of ChIP experiments with Hep3B cells, we carried out QPCR on immunoprecipitated DNA with a primer pair designed for a sequence within CD59 gene that does not bind p53 (negative/ChIP, Table 2). Amplification gave insignificant number of copies compared to that in genomic DNA in the same reaction (1380-fold less, data not shown), confirming very low background in our ChIP experiments.

Discussion

The CD59 gene consists of four exons (50), a 5'-flanking region containing the gene promoter and an enhancer located in intron 1 (nucleotides -1155 to -888 upstream of exon 2) (51). The gene contains two putative p53-responsive elements that do not match exactly the p53-consensus binding sequence (32 and Fig. 1*A*). We first showed by EMSA that these putative p53-responsive motifs are capable of binding p53 *in vitro* (Fig. 1*B*), which is in agreement with the recently published prediction that the CD59 gene possesses functional binding sites for the p53 tumour suppressor protein (52). This

prediction was based on experiments *in vitro* with reporter gene constructs (secreted placental alkaline phosphatase) containing the putative p53-responsive sequences from the CD59 gene. In this paper we have further addressed the role of CD59.1 and CD59.2 putative responsive elements within the CD59 gene in living cells (Fig. 2) by knocking down p53 expression by siRNA. Our findings unambiguously showed that p53 is required for high expression of CD59 in HeLa cells, confirming a functional role for these two elements.

Many tumours initially respond to chemotherapy, however, the surviving cancer cells frequently relapse into a multidrug resistant state (53, 54). Despite the better survival of cancer patients when camptothecin analogues have been used as second-line therapy (55), our experiments suggested that surviving cancer cells after such treatment have even greater resistance to C attack (Fig. 3). This is at least in part a result of increased expression of the CReg protein CD59 in surviving cells after the camptothecin treatment. We showed that the expression level of p53 was increased in surviving cells but also, to a lesser degree, in apoptotic cells that express less CD59 (Figs. 3, 4). These data did not fit a simple relationship between p53 expression and CD59 levels.

In an attempt to resolve this inconsistency we examined the role of post-translational modifications of p53 in regulation of CD59 expression. Recently it was demonstrated that acetylation of p53 may contribute, at least in part, to full activation of specific p53 target genes *in vivo* by increasing its site-specific DNA binding activity (56). Using antibody specific for acetylated p53 we showed that changes in CD59 protein expression in

surviving and apoptotic neuroblastoma cells treated with camptothecin correlated with the level of acetylation of p53 compared to that in untreated cells (Figs. 3, 4). Acetylation of p53 resulted in a better binding of this protein to its responsive elements within the CD59 gene in living cells and to increased CD59 mRNA expression. Decreased acetylation of p53 in apoptotic cells led to approximately 7-fold lower number of p53 molecules bound to its responsive elements compared to surviving cells and consequently to lower expression of CD59. The lower acetylation of p53 in apoptotic cells might be a result of activation of histone deacetylase SIR2 that recently was demonstrated to be a negative regulator of p53 function capable of modulating cellular senescence (57).

Recently it was suggested that p53 might be involved in regulation of CD59 expression in inflammation thus protecting host cells from innate C lysis (32). Our experiments with inflammatory cytokines (Figs. 5, 6) suggest a similar mechanism for regulation of CD59 expression in inflammation as in apoptosis. Hep3B cells were found to modulate their CD59 mRNA expression in response to IFN-γ by decreased acetylation of p53 (Fig. 5). Taken into account that IFN-γ is produced by cells in response primarily to viral infection (45), this may be a defensive mechanism that allows infected host cells to be targeted by C. However, when we treated cells with IL-8, a cytokine that is secreted in acute inflammation (45), we detected higher acetylation of p53 and increased expression of CD59 (Figs. 5, 6). Acetylation of p53 regulates stability as well as its physical interactions with transcriptional coactivators such as p300 (58-60). This might explain the discrepancy between expression level of p53 at mRNA level and at protein level in Hep3B cells treated with IFN-γ (Fig. 5).

Taken together, the data indicate that alterations in p53 levels and acetylation status regulate CD59 expression in cells. Acetylation of p53 may act through multiple mechanisms, including effects on DNA binding, protein stability and protein-protein interactions, to synergistically regulate transcription *in vivo*. Therefore, p53 itself, its positive and negative regulators such as p300 and SIR2, or other mediators of acetylation could be good targets for modulation of CD59 expression in order to facilitate targeting of tumour cells by C.

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References

1. Walport MJ. Complement. First of two parts. N Engl J Med 2001;344:1058–66.
2. Magyarlaki T, Mosolits S, Baranyay F, Buzogany I. Immunohistochemistry of complement response on human renal cell carcinoma biopsies. Tumori 1996;82:473–9.
3. Lucas SD, Karlsson-Parra A, Nilsson B, et al. Tumour-specific deposition of immunoglobulin G and complement in papillary thyroid carcinoma. Hum Pathol 1996;27:1329–35.
4. Matsumoto M, Takeda J, Inoue N, et al. A novel protein that participates in nonself discrimination of malignant cells by homologous complement. Nat Med 1997;3:1266-70.
5. Niculescu F, Rus HG, Retegan M, Vlaicu R. Persistent complement activation on tumour cells in breast cancer. Am J Pathol 1992;140:1039–43.
6. Yamakawa M, Yamada K, Tsuge T, et al. Protection of thyroid cancer cells by complement-regulatory factors. Cancer 1994;73:2808–17.
7. Bjørge L, Hakulinen J, Wahlstrom T, Matre R, Meri S. Complementregulatory proteins in ovarian malignancies. Int J Cancer 1997;70:14–25.
8. Gorter A, Meri S. Immune evasion of tumour cells using membranebound complement regulatory proteins. Immunol Today 1999;20:576–82.
9. Jurianz K, Ziegler S, Garcia-Schuler H, et al. Complement resistance of tumour cells: basal and induced mechanisms. Mol Immunol 1999;36:929–39.
10. Morgan BP, Meri S. Membrane proteins that protect against complement lysis. Springer Semin Immunopathol 1994;15:369–96.

11. Hofman P, Hsi BL, Manie S, Fenichel P, Thyss A, Rossi B. High expression of the antigen recognized by the monoclonal antibody GB24 on human breast carcinomas: a preventive mechanism of malignant tumor cells against complement attack? Breast Cancer Res Treat 1994;32:213–19.

12. Jarvis GA, Li J, Hakulinen J, et al. Expression and function of the complement membrane attack complex inhibitor protectin (CD59) in human prostate cancer. Int J Cancer 1997;71:1049–55.

13. Rushmere N, Knowlden JM, Gee JMW, et al. Analysis of the level of mRNA expression of the membrane regulators of complement, CD59, CD55 and CD46, in breast cancer. Int. J. Cancer 2004;108:930–6.

14. Varsano S, Rashkovsky L, Shapiro H, Ophir D, Bentankur T. Human lung cancer cell lines express cell membrane complement inhibitory proteins and are extremely resistant to complement-mediated lysis: a comparison with normal human respiratory epithelium in vitro, and an insight into mechanism(s) of resistance. Clin Exp Immunol 1998;113:173–82.

15. Maio M, Brasoveanu LI, Coral S, et al. Structure, distribution, and functional role of protectin (CD59) in complement-susceptibility and in immunotherapy of human malignancies. Int J Oncol 1998;13:305–18.

16. el-Deiry WS, Kern SE, Pietenpol JA, Kinzler KW, Vogelstein B. Definition of a consensus binding site for p53. Nature Genet 1992;1:45-9.

17. el-Deiry WS, Tokino T, Velculescu VE, et al. WAF1 a potential mediator of p53 tumour suppression. Cell 1993;75:817-25.

18. Zauberman A, Flusberg D, Haupt Y, Barak Y, Oren M. A functional p53-responsive intronic promoter is contained within the human mdm2 gene. Nucleic Acids Res 1995;23:2584-92.

19. Kastan MB, Zhan Q, el-Deiry WS, et al. A mammalian cell cycle checkpoint pathway utilizing p53 and GADD45 is defective in ataxia-telangiectasia. Cell 1992;71:587-97.

20. Miyashita T, Reeed JC. Tumour suppressor p53 is a direct transcriptional activator of the human bax gene. Cell 1995;80:293-9.

21. Zauberman A, Lubp A, Oren M. Identification of p53 target genes through immune selection of genomic DNA: The cyclin G gene contains two distinct p53 binding sites. Oncogene 1995;10:2361-6.

22. Chen X, Bargonetti J, Prives C. p53 through p21 (WAF1/CIP1) induces cyclin D1 synthesis. Cancer Res 1995;55:4257-63.

23. Buckbinder L, Talbott R, Velasco-Miquel S, et al. Induction of the growth inhibitor IGF-binding protein 3 by p53. Nature 1995;377:646-9.

24. Morris GF, Bischoff JR, Mathews MB. Transcriptional activation of the human proliferating-cell nuclear antigen promoter by p53. Proc Natl Acad Sci 1996;93:895-9.

25. Shin T, Paterson AJ, Kudlow JE. p53 stimulates transcription from the human transforming growth factor-a promoter: a potential growth-stimulatory role for p53. Mol Cell Biol 1995;15:4694-701.

26. Spandidos DA, Zoumpourlis V, Zachos G, Toas SH, Halazonetis TD. Specific recognition of a transcriptional element within the human H-ras proto-oncogene by the p53 tumor suppressor. Int J Oncol 1995;7:1029-34.

27. Deffie A, Wu H, Reinke V, Lozano G. The tumor suppressor p53 regulates its own transcription. Mol Cell Biol 1993;13:3415-23.

28. Furuhata T, Tokino T, Urano T, Nakamura Y. Isolation of a novel GPI-anchored gene specifically regulated by p53; correlation between its expression and anti-cancer drug sensitivity. Oncogene 1996;13:1965-70.

29. Kagawa K, Inove T, Tokino T, Nakamura Y, Akiyama T. Overexpression of GML promotes radiation-induced cell cycle arrest and apoptosis. Biochem Biophys Res Commun 1997;241:481-5.

30. Ueda K, Miyoshi Y, Tokino T, Watatani M, Nakamura Y. Induction of apoptosis in T98G glioblastoma cells by transfection of GML, a target gene. Oncol Res 1999;11:125-32.

31. Kimura Y, Furuhata T, Urano T, Hirata K, Nakamura Y, Tokino T. Genomic structure and chromosomal localization of GML (GPI-anchored molecule-like protein), a gene induced by p53. Genomics 1997;41:477-80.

32. Sampaziotis F, Kokotas S, Gorgoulis V. p53 possibly upregulates the expression of CD58 (LFA-3) and CD59 (MIRL). Med Hypoth 2001;58:136-40.

33. Nalca A, Rangnekar VM. The G1-phase growth-arresting action of interleukin-1 is independent of p53 and p21/WAF1 function. J Biol Chem 1998;273:30517-23.

34. Gotlieb WH, Watson JM, Rezai A, Johnson M, Martinez-Maza O, Berek JS. Cytokine-induced modulation of tumor suppressor gene expression in ovarian cancer cells: up-regulation of p53 gene expression and induction of apoptosis by tumor necrosis factor-alpha. Am J Obstet Gynecol 1994;170:1121-8.

35. Ma W, Pobe JS. Human endothelial cells effectively costimulate cytokine production by, but not differentiation of, naïve CD4+ T cells. J Immunol 1998;131:611-6.

36. Takami A, Zeng W, Wang H, Matsuda T, Nakao S. Cytotoxicity against lymphoblastoid cells mediated by a T-cell clone from an aplastic anaemia patient: role of CD59 on target cells. Br J Haematol 1999;107:791-6.

37. Liversidge J, Dawson R, Hoey S, McKay D, Grabowski P, Forrester JV. CD59 and CD48 expressed by rat retinal pigment epithelial cells are major ligands for the CD2-mediated alternative pathway of T cell activation. J Immunol 1996;156:3696-703.

38. Orlando V, Paro, R. Mapping Polycomb-repressed domains in the bithorax complex using in vivo formaldehyde cross-linked chromatin. Cell 1993;75:1187–98.

39. Donev R, Horton R, Beck S, et al. Recruitment of heterogeneous nuclear ribonucleoprotein A1 in vivo to the LMP/TAP region of the major histocompatibility complex. J Biol Chem 2003;278:5214-26.

40. De Song X, Sheppard HM, Norman AW, Liu X. Mitogen-activated protein kinase is involved in the degradation of p53 protein in bryostatin-1-induced differentiation of the acute promyelocytic leukemia NB4 cell line. J Biol Chem 1999;274:1677-82.

41. Sessa C, Wanders J, Roelvink M, et al. Second-line treatment of small-cell lung cancer with the camptothecin-derivative GI147211: a study of the EORTC Early Clinical Studies Group (ECSG). Annals of Oncology 2000;11:207-10.

42. Kwong MS, Bleickardt E, Murren JR. Camptothecin and taxane regimens for small-cell lung cancer. Oncology (Huntingt) 2002;16:33-8.

43. Cole DS, Morgan BP. Functional significance of complement activation and complement regulator loss on apoptotic neuroblastoma cells. Immunol 2004;113,75.

44. van Beek J, van Meurs M, 'tHart BA, et al. Decay-accelerating factor (CD55) is expressed by neurons in response to chronic but not acute autoimmune central nervous system inflammation associated with complement activation. J Immunol 2005;174:2353-65.

45. Feghali CA, Wright TM. Cytokines in acute and chronic inflammation. Frontiers Biosci 1997;2:12-26.

46. Spiller OB, Criado-Garcia O, Rodriguez De Cordoba S, Morgan BP. Cytokine-mediated up-regulation of CD55 and CD59 protects human hepatoma cells from complement attack. Clin Exp Immunol 2000;121:234-41.

47. Wolf D, Rotter V. Major deletions in the gene encoding the p53 tumor antigen cause lack of p53 expression in HL-60 cells. Proc Natl Acad Sci USA 1985;82:790-4.

48. Petryshyn R, Chen JJ, Danley L, Matts RL. Effect of interferon on protein translation during growth stages of 3T3 cells. Arch Biochem Biophys 1996;326:290-7.

49. Hiremath MM, Mikhael AI, Taylor LS, Musso T, McVicar DW. Complex regulation of the Csk homologous kinase (Chk) by IL-4 family cytokines and IFN-gamma in human peripheral blood monocytes. Mol Immunol 2004;41:901-10.

50. Holguin MH, Martin CB, Eggett T, Parker CJ. Analysis of the gene that encodes the complement regulatory protein, membrane inhibitor of reactive lysis (CD59). J Immunol 1996;157:1659-68.

51. Tone M, Diamond LE, Walsh LA, et al. High level transcription of the complement regulatory protein CD59 requires an enhancer located in intron 1. J Biol Chem 1999;274:710-6.

52. Gazouli M, Kokotas S, Zoumpourlis V, et al. The complement inhibitor CD59 and the lymphocyte function-associated antigen-3 (LFA-3, CD58) genes possess functional binding sites for the p53 tumor suppressor protein. Anticancer Res. 2002;22:4237-41.

53. Goto H, Keshelava N, Matthay K, et al. Multidrug Resistance-Associated Protein 1 (MRP1) Expression in Neuroblastoma Cell Lines and Primary Tumors. Med Pediatric Oncol 2000;35:619-22.

54. Filips M, Pohl G, Rudfas M, et al. Clinical Role of Multidrug Resistance Protein 1 Expression in Chemotherapy Resistance in Early-Stage Breast Cancer: The Austrian Breast and Colorectal Cancer Study Group. J Clinical Oncol 2005;23:1161-8.

55. Kwong MS, Bleickardt E, Murren JR. Camptothecin and taxane regimens for small-cell lung cancer. Oncology (Huntingt) 2002;16:33-8.

56. Luo JL, Li M, Tang Y, Laszkowska M, Roeder RG, Gu W. Acetylation of p53 augments its site-specific DNA binding both in vitro and in vivo. Proc Natl Acad Sci USA 2004;101:2259-64.

57. Langley E, Pearson M, Faretta M, et al. Human SIR2 deacetylates p53 and antagonizes PML/p53-induced cellular senescence. EMBO J 2002;21:2383-96.

58. Brooks CL, Gu W. Ubiquitination, phosphorylation and acetylation: the molecular basis for p53 regulation. Curr Opin Cell Biol 2003;15:164-71.

59. Barlev NA, Liu L, Chehab NH, et al. Acetylation of p53 activates transcription through recruitment of coactivators/histone acetyltransferases. Mol Cell 2001;8:1243-54.

60. Li M, Luo J, Brooks C, Gu W. Acetylation of p53 inhibits its ubiquitination by Mdm2. J Biol Chem 2002;277:50607-11.

Figure Legends

Figure 1. **p53 binds to the putative binding sites within the CD59 gene *in vitro*.** *A,* CD59.1, the first putative p53-responsive element, is located in the 5' flanking region of the CD59 gene ranging from nucleotide -1665 to -1637 upstream of exon 1. CD59.2 is the second putative p53-binding sequence located in intron 1 of the CD59 ranging from nucleotide –675 to –645 upstream of exon 2. Possible p53 half-binding sites are marked in capital letters. The differences between these and the p53 consensus binding sequence are underlined. *B,* EMSA with samples labelled with horse radish peroxidase (HRP), incubated with 100 ng wild type recombinant p53 in the presence of 0.1 μg/μl poly-d(IC). Oligonucleotides were separated in a 2% agarose gel, and detected by autoradiography.

Figure 2. **p53 dependant expression of CD59 in HeLa cells.** *A,* Western blot analysis of p53 (anti-p53, cloneBP53-12 recognises all p53 modifications) and CD59 expression in HeLa cells and cells 36h after transfection with siRNA specifically blocking expression of p53. Detection of p42 MAPK was performed as a control for the siRNA specificity. *B,* FACS analysis of CD59 expression in HeLa and p53siRNA transfected cells.

Figure 3. **Alterations in CD59 expression in IMR32 cells treated with camptothecin.** *A,* Western blot analysis of CD59 expression in IMR32 cells treated with camptothecin for either 24 or 48h. *B,* Expression of CD59 in IMR32 cells treated with camptothecin for 24 or 48h detected by QPCR. Expression in untreated cells is set as 100%. Values are mean ±SD for two independent experiments. Compared sets are shown by bars with interrelated P values for the comparison. *C,* Resistance of IMR32 (-◆-) and surviving

IMR32 (-■-) cells to C lysis. Lysis assay with pre-incubation of both untreated (-●-) and surviving (-▲-) IMR32 cells with CD59-blocking antibody BRIC229 was carried out as a control. Values are mean ±SD for three independent experiments.

Figure 4. **Expression of p53 and recruitment to the CD59 gene.** *A,* Two different antibodies were used for detection of p53: anti-p53, clone BP53-12 recognising all forms of p53 and anti-acetyl-p53 (Lys373, Lys382) to detect the changes in p53 acetylation. *B,* IMR32 cells were treated with captothecin for either 24 or 48h. Immunoprecipitation was performed with anti-acetyl-p53 (Lys373, Lys382) antibody. Binding of p53 to its responsive elements CD59.1 (black bars) and CD59.2 (striped bars) in control IMR32 cells is set as 1. Values are mean ±SD for two independent ChIP experiments each analyzed in duplicate. Compared sets are shown by bars with interrelated P values for the comparison.

Figure 5. **Effect of cytokines on p53 and CD59 expression in IMR32 and Hep3B cells.** *A,* QPCR analysis of p53 (black bars) and CD59 (white bars) expression following an incubation of Hep3B and HL60 cells with IFN-γ or IL-8. Results from two independent measurements displayed a significant difference between untreated and cytokine treated Hep3B samples (*$p < 0.05$, **$p < 0.01$). However, no significant difference was observed between HL60 samples. *B,* Flow cytometry analysis of p53 (black bars), (Lys373, Lys382) acetylated p53 (white bars) and CD59 (striped bars) following the same treatments as in *A* (*$p < 0.01$, **$p < 0.001$). Data graphed represent the mean ±SD of four measurements obtained from two separate experiments.

Figure 6. **ChIP analysis of recruitment of p53 to the CD59 gene in living Hep3B cells.** Cells were treated with IFN-γ or IL-8 for 36h. Immunoprecipitation was carried out either with anti-acetyl-p53 (Lys373, Lys382) *(A)* or anti-p53, clone BP53-12 *(B)* antibodies. Binding of p53 to its responsive elements CD59.1 (black bars) and CD59.2 (white bars) in untreated cells is set as 1. Values are mean ±SD for two independent ChIP experiments each analyzed in duplicate. Compared sets are shown by bars with interrelated P values for the comparison.

Table 1. Deoxyoligonucleotides used in EMSA.

Sequence name	Sequence
CD59.1	5'-TGTTTTAAGGAGACATGCTTTAAATATCAAAGCAAGTCATCCTGATTTAA-3'
CD59.1-complementary	5'-TTAAATCAGGATGACTTGCTTTGATATTTAAAGCATGTCTCCTTAAAACA-3'
CD59.2	5'-TACTTAGGCAAGTTCAAAACCTCCCTAAGCTTTGGCTTCTT-3'
CD59.2-complementary	5'-AAGAAGCCAAAGCTTAGGGAGGTTTTGAACTTGCCTAAGTA-3'
Positive control	5'-TGTTTTAAGGAGACATGCTTTAAATATCAAAGCAAGTCTTCCTGATTTAA-3'
Positive control-complementary	5'-TTAAATCAGGAAGACTTGCTTTGATATTTAAAGCATGTCTCCTTAAAACA-3'
Negative control	5'-TGTTTTAAGGCTCACGTAGGTAAATATCACTCACGTAGGTCCTGATTTAA-3'
Negative control-complementary	5'-TTAAATCAGGACCTACGTGAG TGATATTTACCTACGTGAGCCTTAAAACA-3'

Table 2. Primer pairs used for real time quantitative PCR/RT-PCR

Sequence name	Primers	Primer concentrations, μM
β-actin	5'-GGATCAGCAAGCAGGAGTATGA-3'(F)	900/50
	5'-GCGCAAGTTAGGTTTTGTCAAG-3' (R)	
p53	5'-GCGTGAGCGCTTCGAGAT-3' (F)	300/900
	5'-AGCCTGGGCATCCTTGAGT-3' (R)	
CD59	5'-TAACCCAACTGCTGACTGCAA-3' (F)	50/300
	5'-TTTGGTAATGAGACACGCATCAA-3' (R)	
CD59.1/ChIP	5'-GATGTCAGGGAATGAGTTCC-3' (F)	300/300
	5'-GAGCTTTGGAATCCATCTTGG-3' (R)	
CD59.2/ChIP	5'-TCCAACCCTACTTTACCCAG-3' (F)	300/900
	5'-TGTAGGATTGGAGTTGGGAG-3' (R)	
Negative/ChIP	5'-CATTGGAGTGTGGCTACAGT-3' (F)	300/300
	5'-AGCTTCTCAGGTGTGAGGCT-3' (R)	

A

CD59.1

Pu-Pu-Pu-C-A/T-A/T-G-Py-Py-Py Pu-Pu-Pu-C-A/T-A/T-G-Py-Py-Py

A G A C A T G C T T taaatatca A A G C A A G T C A

-1665 -1637

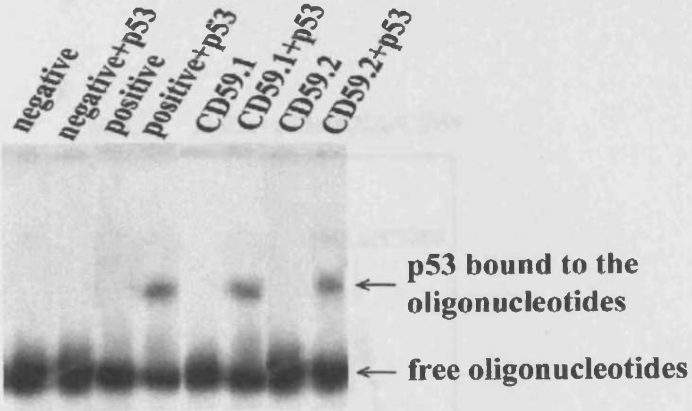
CD59.2

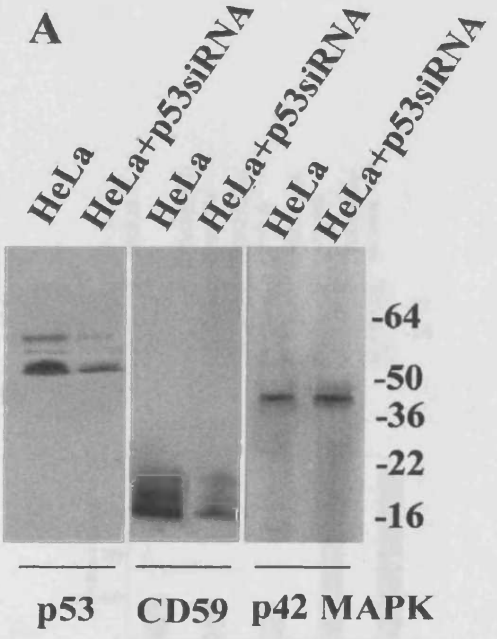
Pu-Pu-Pu-C-A/T-A/T-G-Py-Py-Py Pu-Pu-Pu-C-A/T-A/T-G-Py-Py-Py Pu-Pu-Pu-C-A/T-A/T-G-Py-Py-Py

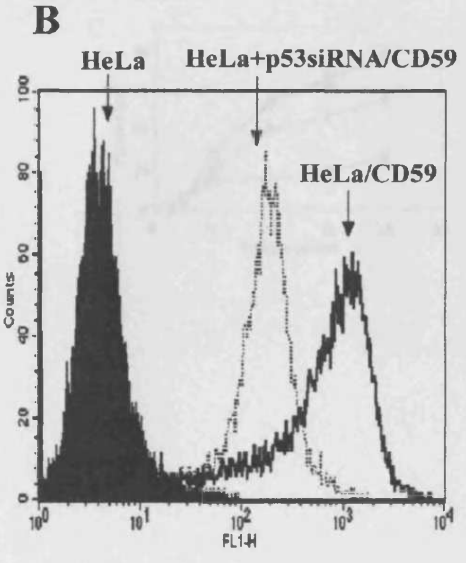
A G G C A A G T T CaA A A C C T C C C T A A G C T T T G G C

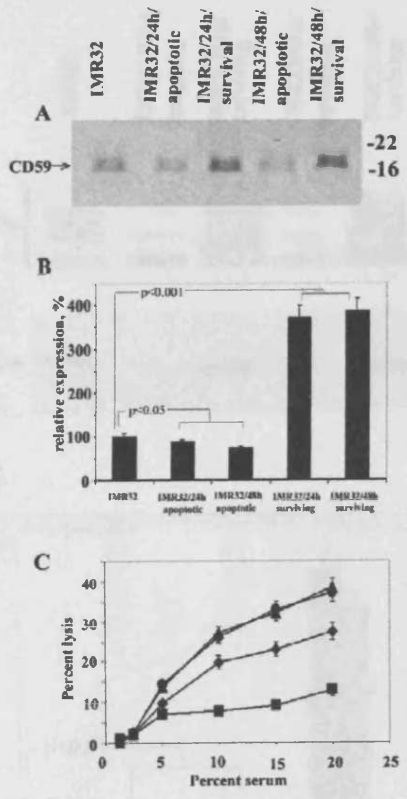
-675 -645

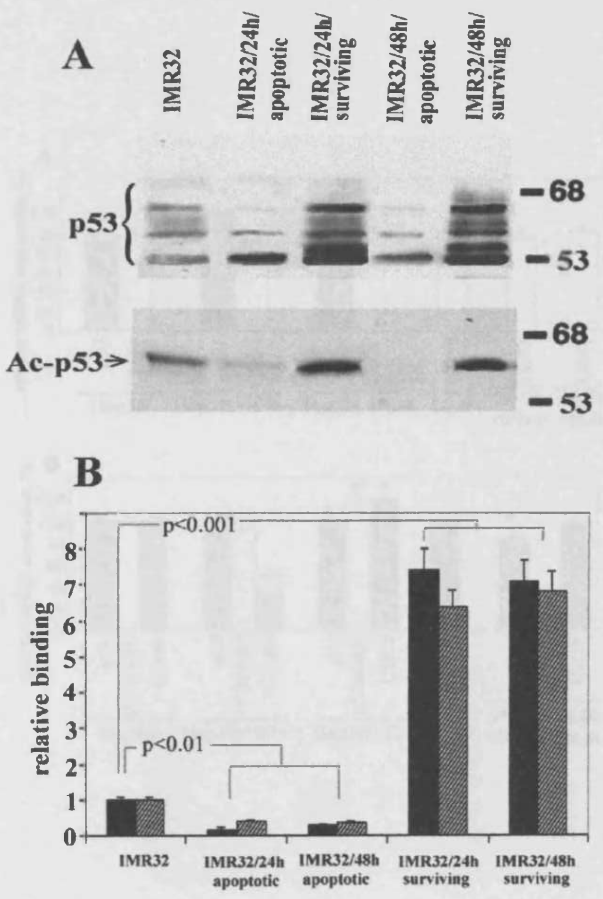
B




A
HeLa
HeLa+p53siRNA
HeLa
HeLa+p53siRNA
HeLa
HeLa+p53siRNA
-64
-50
-36
-22
-16
p53
CD59
p42 MAPK

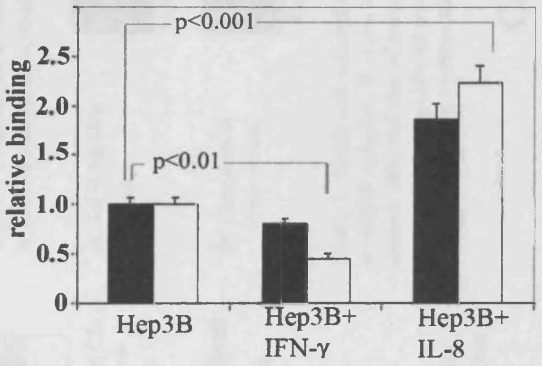

B
HeLa
HeLa+p53siRNA/CD59
HeLa/CD59
Counts
FL1-H


A
IMR32
IMR32/24h/ apoptotic
IMR32/24h/ survival
IMR32/48h/ apoptotic
IMR32/48h/ survival
CD59
-22
-16
B
relative expression, %
p<0.001
p<0.05
C
Percent lysis
Percent serum

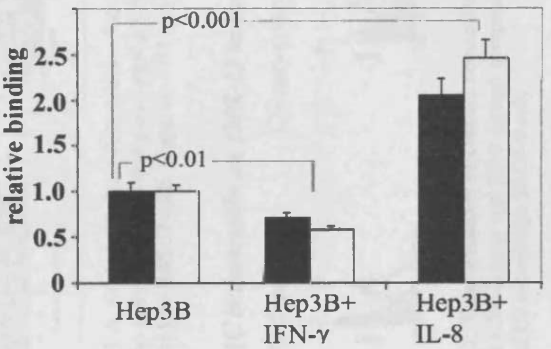

A
IMR32
IMR32/24h/ apoptotic
IMR32/24h/ surviving
IMR32/48h/ apoptotic
IMR32/48h/ surviving
p53
68
53
Ac-p53
68
53
B
relative binding
p<0.001
p<0.01
0
1
2
3
4
5
6
7
8
IMR32
IMR32/24h apoptotic
IMR32/48h apoptotic
IMR32/24h surviving
IMR32/48h surviving

A
relative mRNA expression, %
0
20
40
60
80
100
120
Hep3B
Hep3B+IFN-γ
Hep3B+IL-8
HL60
HL60 +IFN-γ
HL60 +IL-8
p53
CD59
**
*
B
relative protein expression, %
0
20
40
60
80
100
120
Hep3B
Hep3B+IFN-γ
Hep3B+IL-8
HL60
HL60 +IFN-γ
HL60 +IL-8
p53
p53acetyl
CD59
**
*

A


p<0.001
p<0.01
relative binding
2.5
2.0
1.5
1.0
0.5
0
Hep3B
Hep3B+ IFN-γ
Hep3B+ IL-8

B


p<0.001
p<0.01
relative binding
2.5
2.0
1.5
1.0
0.5
0
Hep3B
Hep3B+ IFN-γ
Hep3B+ IL-8

Non-lethal events in IMR-32 neuroblastoma cells attacked by the complement Membrane Attack Complex

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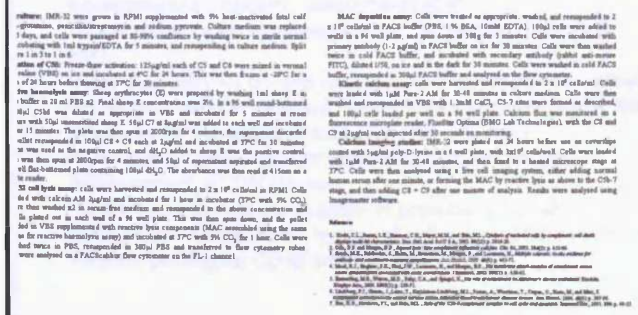
Introduction

mplement is an important component of the innate mune system. The pore-forming membrane attack mplex (MAC) comprises the terminal pathway of the mplement system, and has long been know to cause lysis cells on which it is deposited[1]. It is now known that the AC does more than just kill cells. In sub-lytic doses, it can ivate cells, cause the release of inflammatory mediators, luce protection against lytic complement attack, induce oliferation, and modulate apoptosis[2].

the central nervous system, the MAC has been implicated the pathogenesis of multiple sclerosis[3, 4], Alzheimer's ease[5], and stroke[6]. The main focus so far has been on : damaging effects of the MAC, but work on godendrocytes has indicated that in this cell type sub-lytic AC has a role in promoting repair[7]. We chose to study se effects in neurons as their death results in permanent urological deficit in vivo. In this study we aimed to define tial non-lethal events caused by the MAC in a neuron-rived cell line using kinetic calcium assays and single cell aging techniques

Materials and Methods



Results

1) IMR-32 are resistant to MAC mediated lysis

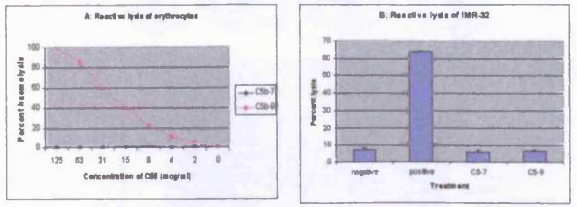


Figure 1 A: Erythrocyte lysis demonstrates functional activity of C5-9, but no activity with the non-pore forming C5-7. B: C5-9 attack is unable to lyse IMR-32 cells. Negative: VBS only. Positive: serum 1/5.

2) MAC is detectable on IMR-32 in the absence of lysis

C7 deposition C9 neo-epitope deposition

Figure 2: Flow cytometry of IMR-32 demonstrating C7 deposition on both C5-7 (black) and C5-9 (green) compared with control (red fill), and C9 neo-epitope on C5-9 only.

3) Sub-lytic MAC causes calcium flux in IMR-32

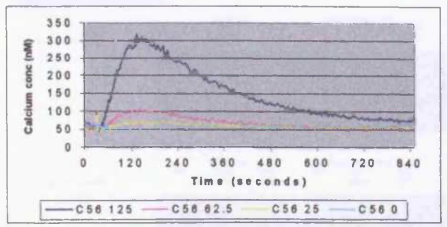


Figure 3: Calcium flux in IMR-32 during reactive lysis. Cells are coated with C5-7; at 30seconds C8 and C9 are added. A dose-response effect is seen. C56 concentrations in mcg/ml.

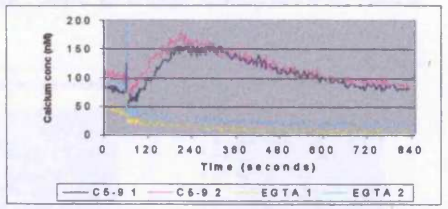
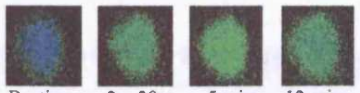


Figure 4: Calcium flux is abolished with the addition of 10mM EGTA in IMR-32 reactive lysis, indicating calcium stores are not involved.

A: Sub-lytic MAC attack



B: Complement lysis (serum)

Figure 5: Single cell calcium imaging. A: Sublytic MAC attack shows a reversible response. B: Lytic attack is slower, and results in irreversible calcium entry and loss of membrane integrity. Addition of C8 and C9 (for A) or normal serum (for B) was at 1 minute. Pseudocolour scale: dark blue – light blue – green – yellow with increasing calcium concentration.

Conclusion

These results demonstrate that sub-lytic MAC is able to induce a non-lethal event, reversible calcium influx, in IMR-32 neuroblastoma cells. This is dependant on extracellular calcium entry. The work extends such observations to a neuron-derived cell type, and provides a system for further characterising sublytic MAC-mediated phenotypic changes in these cells, such as modulation of apoptosis and alteration of cell surface molecules involved in controlling the brain's immune response. Such events may be of importance in damage limitation and repair in inflammatory disorders of the central nervous system.

Functional significance of complement regulator loss on apoptotic neuroblastoma cells

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Background

The safe disposal of apoptotic cells is a crucial process in the central nervous system in both health and disease. Failure to clear apoptotic debris may provoke a damaging inflammatory reaction and exacerbate pathology[1,2]. Complement has been shown to play a role in clearing such debris by opsonisation of apoptotic cells[2,3], but complement is also known to be pro-inflammatory and to cause necrotic cell death by insertion of the membrane attack complex (MAC)[4].Self-cells are normally protected from complement activation by the membrane regulators CD46 and CD55 controlling the C3 convertase, and CD59 controlling MAC formation. CD55 and CD59 are known to be lost during neutrophil apoptosis[5].

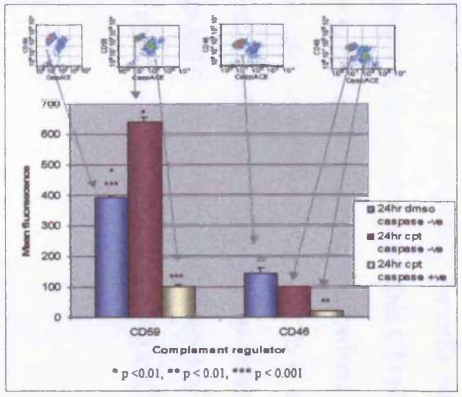
Questions:

1. **Does complement regulator expression change during apoptosis of neuronal cells?**
2. **What are the consequences for the apoptotic cell: are complement deposition and susceptibility to lysis altered?**

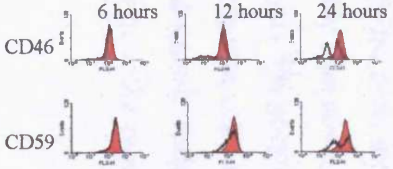
Methods

- Induction of apoptosis in IMR-32 neuroblastoma cells with the topoisomerase I inhibitor camptothecin (CPT).
- Flow cytometry for apoptosis marker CaspACE (FITC-VAD-fmk).
- Flow cytometry for complement regulator levels on cell surface.
- Incubation with C8 depleted human serum and analysis by flow cytometry for complement activation and deposition.
- Lysis assay: incubation with normal human serum and measurement of LDH release.
- Trichloroacetic acid precipitation of proteins shed into culture supernatant; NP40 lysates of cell layers. SDS-PAGE and Western blotting for CD46 and CD59.

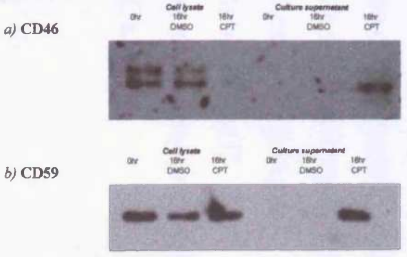
1. Apoptotic IMR-32 lose the complement regulators CD46 and CD59



2. Complement regulators begin to be lost 12 hours after induction of apoptosis

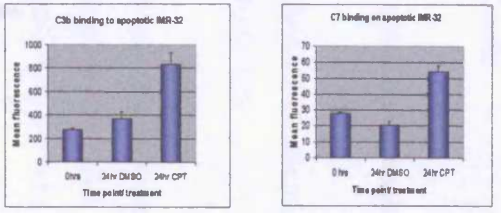


3. CD46 and CD59 are shed into the culture supernatant

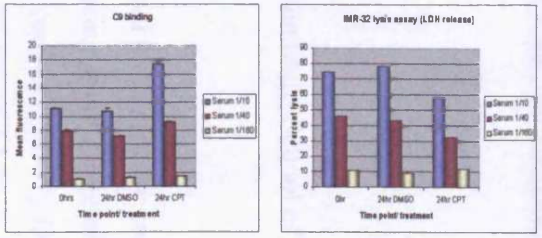


CD46 is lost from the camptothecin treated cells, and a lower MW form appears in the supernatant. In contrast, the camptothecin treated cells still have CD59 despite its appearance in the supernatant.

4. Apoptotic cells activate complement more readily



5. Apoptotic cells bind more C9 but are more resistant to lysis



Discussion:

Apoptotic neuroblastoma cells lose CD46 and CD59 once caspases are activated, shedding them into the surrounding medium. The lower MW of shed CD46 suggests a cleavage event may be responsible. Of note, caspase negative cells retain high levels of CD59, but the significance of this is not known. There is an increase in complement activation on apoptotic cells, and despite increased C9 binding they are more resistant to lysis. We are currently investigating the mechanism of shedding and the role of specific caspases in CD46 and CD59 loss.

References:
1. Savill, J. and Fadok V. (2000) Nature 407:748-788; 2. Taylor P.R. et al (2000) J. Exp. Med. 192:359-366; 3. Botto, M. et al (1998) Nat. Genetics 19: 56-59; 4. Morgan, B.P. (1990) Academic Press, London; 5. Jones J. and Morgan B.P. (1995) Immunology 86:651-660.

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Abstract for BSI 2004

Functional significance of complement activation and complement regulator loss on apoptotic neuroblastoma cells

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The safe disposal of apoptotic cells is a crucial process in the central nervous system in both health and disease. Complement (C) has a role in clearing apoptotic debris, but is also known to cause necrotic cell death by insertion of the membrane attack complex (MAC). Our aim was to study whether C regulator expression and susceptibility to C activation and lysis is altered on neuronal cells undergoing apoptosis. We have demonstrated that apoptotic IMR-32 neuroblastoma cells lose the C regulators CD46 and CD59. This occurred only on cells positive for apoptotic markers, and was caspase dependent. Western blotting revealed that CD46 and CD59 had been shed into the culture supernatant. Apoptotic cells activated C and were opsonised more readily than control cells. Despite binding more MAC, apoptotic cells were more resistant to C mediated lysis. Phagocytosis studies are ongoing to determine the effects of these processes on clearance of apoptotic cells. Our studies suggest C activation on apoptotic neuronal cells promotes their safe disposal, while changes in the apoptotic cell, independent of classical C regulators, protect it from necrosis in the face of MAC attack.