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# Expression and function of membrane regulators of complement on rat astrocytes in culture

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## SUMMARY

Human astrocytes express CD59, decay accelerating factor and membrane cofactor protein to restrict the damaging effect of complement (C) activation on their cell surface. 5I2 antigen (5I2 Ag) is the functional analogue of the latter two proteins in rats. We here demonstrate the surface expression on rat astrocytes of CD59 and 5I2 Ag and use sodium dodecyl sulphate–polyacrylamide gel electrophoresis and Western blotting to confirm their identity and to quantify expression. Rat CD59 (MW 20 000) was expressed at $720 \times 10^3$ molecules per cell and 5I2 Ag (MW 58 000 and 64 000) at $625 \times 10^3$ molecules per cell. Reverse transcription–polymerase chain reaction using specific oligonucleotide primers demonstrated expression of mRNA for each protein. Twenty-four-hour stimulation with inflammatory cytokines (interferon-$\gamma$, tumour necrosis factor-$\alpha$, interleukins-1$\beta$, -2 and -6) or phorbol myristate acetate had no significant effect on the level of expression of either protein as determined by Western blotting. Lysis caused by classical pathway activation of C in human or rat serum was enhanced by blocking the function of CD59 and 5I2 Ag on rat astrocytes with monoclonal antibodies.

## INTRODUCTION

Activation of complement (C) is implicated in the pathogenesis of various diseases of the central nervous system (CNS) (reviewed in ref. 1) including multiple sclerosis (MS)[2] and Alzheimer's disease[3] where C activation products have been detected within the CNS. There is also evidence that C activation has a pathogenic role in animal models of human neurological diseases. Perivascular C deposition within the CNS is observed in experimental allergic encephalomyelitis (EAE) in Lewis rats and depletion of C using cobra venom factor (CVF) or inhibition using soluble complement receptor 1 (CR1, CD35) delays or suppresses symptoms of the disease in this animal model of MS.[4,5] There is evidence from *in vitro* studies that some CNS cells are particularly susceptible to the destructive action of homologous C. For example, oligodendrocytes, the myelin-forming cells of the CNS, directly activate the classical pathway (CP) of C and are extremely sensitive to the lytic action of homologous C.[6,7] Neurons are also susceptible to C damage and it has been suggested by some workers that the killing of neurons in Alzheimer's disease is triggered by activation of C on the beta amyloid component $\beta$A4-surrounding neurons.[8] In contrast to oligodendrocytes and neurons, rat astrocytes have been shown to be resistant to homologous C *in vitro*.[7,9]

Most nucleated cells protect themselves from the potential destructive action of C primarily by the activities of intrinsic membrane regulatory proteins.[10] In humans, decay accelerating factor (DAF, CD55)[11] and membrane cofactor protein (MCP, CD46)[12] function to inhibit C3b deposition on self tissues by respectively accelerating spontaneous decay of the C3- and C5-convertases of both the classical and alternative pathways or by acting as a cofactor for factor I-mediated cleavage of these convertases. CR1 has both the decay acceleration and cofactor activities described above.[13] CD59[14,15] regulates the formation and function of the lytic C5b–9 complex by binding C8 and preventing the unfolding and membrane insertion of C9 and by binding C9 and restricting its polymerization.[16]

In rodents a single protein has been described which mediates both decay-accelerating and cofactor activities; in the

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Abbreviations: C, complement; CNS, central nervous system; CP, classical pathway; CR1, complement receptor 1; DAF decay accelerating factor; DMEM, Dulbecco's modified Eagle's medium; EAE, experimental allergic encephalomyelitis; EBSS, Earle's balanced salt solution; FCS, fetal calf serum; FITC, fluorescein isothiocyanate; GFAP, glial fibrillary acidic protein; GVB, gelatin-veronal-buffered saline; HBSS, Hanks' balanced salt solution; mAb, monoclonal antibody; MCP, membrane cofactor protein; NHS, normal human serum; NRS, normal rat serum; PI, propidium iodide; PLL, poly-L-lysine; PMA, phorbol myristate acetate; RGC, rat glial cell.

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mouse, Crry/p65[17] and in rat, 5I2 Ag.[18,19] These proteins share structural homology with MCP, DAF and each other. Both mouse and rat homologues of CR1 have been described[20,21] but the function of rat CR1 has not been investigated. The rat analogue of CD59 has been described[22] and sequenced,[23] demonstrating its close homology to human CD59.[24]

The tissue distribution of CD59 and 5I2 Ag has been investigated at the immunohistological level in the rat.[25] Both proteins were widely expressed, notably on vascular endothelium and circulating cells. In the nervous system the proteins were expressed on the Schwann sheath of peripheral nerve fibres and on ependymal cells, but were not detected on glial cells and neurons in the CNS. Immunohistological studies suggest that glial cells in the human CNS also lack CD59 or express it very weakly, whereas Schwann cells are CD59 positive.[26–28] In contrast to these histological findings, human astrocytes *in vitro* have been shown to express CD59, MCP and DAF[28–31] but did not express CR1,[29] and rat astrocytes *in vitro* expressed CD59[7] and unidentified proteins that cross-reacted with polyclonal antibodies to human DAF and human MCP.[32]

Here we further investigate expression and function of CD59 and 5I2 Ag on cultured rat astrocytes and demonstrate their potential importance in limiting C-mediated damage to these cells during infection and inflammation.

## MATERIALS AND METHODS

*Antibodies, cytokines, other reagents and buffers*

Monoclonal antibodies (mAb): TH9 and 6D1 mAb (both anti-rat CD59) and 5I2 mAb were from our laboratories and have been described previously.[22,33] IgG fractions of these antibodies were prepared using protein A affinity chromatography. Anti-glial fibrillary acidic protein (GFAP) and anti-galactocerebroside were purchased from Sigma (Dorset, UK). OX-42 mAb (anti-complement receptor 3 [CR3]) was purchased from Seralab Ltd. (Sussex, UK). O4 mAb is described previously.[34]

Polyclonal antibodies: rabbit anti-mouse Crry was from our laboratory and has been described previously.[35] Rabbit anti-GFAP and sheep anti-rat 5′-nucleotidase were gifts from Dr L. Cuzner (London, UK) and Dr J. P. Luzio (Cambridge, UK), respectively.

Conjugated antibodies for FACScan analysis rabbit anti-mouse IgG : fluorescein isothiocyanate (FITC) and goat anti-rabbit IgG : FITC were from Dako Ltd, Bucks., UK and Seralab Ltd, respectively. For fluorescence microscopy goat anti-mouse IgG : FITC and donkey anti-rabbit IgG:rhodamine were from Jackson Immunoresearch Labs Inc, West Grove, PA, USA. For Western blotting peroxidase-conjugated goat anti-mouse IgG and goat anti-rabbit IgG were from Biorad, Stevenage, UK, and peroxidase-conjugated goat anti-rat IgG was from Jackson Immunoresearch.

Cytokines: recombinant human interferon-$\gamma$ (IFN-$\gamma$), tumour necrosis factor-$\alpha$ (TNF-$\alpha$) and interleukin-1$\beta$ (IL1-$\beta$) were a gift from Hoffman la Roche, NJ, USA. Recombinant human IL-2 and IL-6 were a gift from Dr C. Fagin, UWCM, Cardiff, UK.

Other reagents: phorbol myristate acetate (PMA) was from Sigma. Normal human and normal rat serum (NHS and NRS) were prepared from fresh clotted blood and stored frozen at $-70°$ prior to use.

Buffers: phosphate buffered saline (PBS) comprised 137 mM NaCl, 2·7 mM KCl, 8·1 mM $Na_2HPO_4$, and 1·5 mM $NaH_2PO_4$ (pH 7·3). PBSAA comprised PBS containing 1% bovine serum albumin (BSA) and 0·01% sodium azide. Gelatin-veronal-buffered saline (GVB) comprised 2·8 mM barbituric acid, 145·5 mM NaCl, 0·8 mM $MgCl_2$, 0·3 mM $CaCl_2$, 0·9 mM Na sodium, 0·1% gelatin (pH 7·2).

*Cell culture*

Whole brains were dissected from 1–2-day-old Wistar rat pups, the meninges carefully and completely removed and brain tissue minced with a scalpel prior to enzymic digestion. One milliletre of 0·1% trypsin per rat brain was added and incubation was at 37° for 20 min with occasional mixing. After addition of DNAase the digest was centrifuged at 200 *g* for 5 min, the supernatant discarded and the pellet triturated through a Pasteur pipette in Hanks' balanced salt solution (HBSS) containing BSA, trypsin inhibitor and DNAase. This mixture was then plated out at $10^7$ cells/10 ml RGC medium/75 $cm^2$ poly-L-lysine (PLL)-coated flask. Rat glial cell (RGC) medium comprised Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS), 5 IU/ml penicillin and 50 $\mu$g/ml streptomycin. To assess the effects of different concentrations of FCS, some preparations were plated out and grown in RGC containing 0%, 1%, 2·5% or 5% FCS. Medium was changed every other day until day 8–10 when the mixed glial cell cultures typically appeared as a confluent bed of astrocytes underlying a mixed population of cells comprising oligodendrocytes, oligodendrocyte-type 2 astrocyte (O2A) progenitor cells, macrophages and microglia. These cells were removed from the adherent monolayer of astrocytes by shaking at 250 r.p.m. for 18–20 hr at 37° as described by McCarthy and de Vellis.[36] Extensive rinsing of the astrocytes, repeating the overnight shake and harvesting with 2 mg/ml collagenase (type 1A, Sigma) in PBS typically provided astrocyte cultures that were >98% pure as judged by staining for GFAP and with no cells positive for oligodendrocyte (galactocerebroside) or microglial cell (CR3) markers. Astrocytes were also O4-negative, identifying them as type 1 astrocytes. Subsequent passages of cells were grown in RGC medium in 75 $cm^2$ flasks or on glass coverslips (neither PLL-coated). Cells in passage 1–4 were used in subsequent experiments, harvested from culture using collagenase as described above and allowed to recover in RGC medium at 37° with gentle shaking for 2 hr.

*Immunofluorescence studies*

*Flow cytometric analysis.* Rat astrocytes in passage 2 were washed twice in PBSAA and resuspended at $10^6$ cells/ml in the same buffer. Cells (100 $\mu$l) were incubated on ice for 30 min with 100 $\mu$l of one of the following antibodies: TH9 mAb, 6D1 mAb, 5I2 mAb (each at 25 $\mu$g/ml) or rabbit anti-mouse Crry antiserum at 1/500 dilution. Control cells were incubated with 25 $\mu$g/ml mouse or rabbit IgG. After washing three times in PBSAA cells were resuspended in 200 $\mu$l of a 1/100 dilution of the appropriate FITC-conjugated second antibody and incubated for a further 30 min on ice. Cells were then washed three times, fixed in 1% paraformaldehyde in PBSAA and analysed on a FACScan (Becton Dickinson, Oxford, UK). For analysis of the effects of different concentrations of FCS on the expression of the regulators, cells were harvested in passage 1 and stained as above.

*Indirect immunofluorescence microscopy.* Rat astrocytes in passage 1 grown on coverslips were double stained for GFAP and rat CD59 or 5I2 antigen. All washes were performed by dipping coverslips in buffered Earle's balanced salt solution (EBSS) and draining on tissue. All antibody dilutions were made in HBSS containing 10% heat-inactivated FCS and 0·1% sodium azide and all incubations with antibody were for 30 min at 37°. Coverslips were washed and incubated with 100 $\mu$l 5I2 mAb, 6D1 mAb (each at 24 $\mu$g/ml) or rabbit anti-mouse Crry (1/200 of antiserum). After washing, coverslips were incubated with FITC-conjugated anti-mouse IgG or rhodamine-conjugated anti-rabbit IgG as appropriate (1/50). Further washing was followed by fixation for 15 min at 0° in 95% ethanol/5% acetic acid and incubation with anti GFAP mAb (ascites, 1/400) or rabbit anti-GFAP (antiserum, 1/500). Subsequent washing was followed by a final incubation with the relevant fluorescent-conjugated antibody. Final washing, mounting in a drop of Citifluor (Citifluor Products, Canterbury, UK) and sealing on glass microscope slides allowed viewing and photography of cells using an inverted fluorescence photomicroscope (Nikon Ltd, Kingston, UK).

*Cytokine stimulation and Western blotting*

Rat astrocytes in passage 1–4 were rinsed thoroughly in DMEM and incubated for 24 hr in DMEM alone or in DMEM containing one of the following cytokines: IFN-$\gamma$ (200 IU/ml), TNF-$\alpha$ (200 IU/ml), IL-1$\beta$ (100 IU/ml), IL-2 (100 IU/ml), IL-6 (100 IU/ml), or PMA (10 ng/ml). Cell culture supernatants were concentrated tenfold by lyophilization. Cells were harvested by treatment with PBS containing 10 mM EDTA, counted, centrifuged and subsequently lysed on ice in 20 mM Tris–HCl (pH 8·2) containing 40 mM NaCl, 2 mM EDTA and 1% Nonidet P-40. Astrocyte supernatants and lysates were separated by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) under non-reducing conditions and blotted onto nitrocellulose by standard methods. Blots were probed with TH9 mAb (5 $\mu$g/ml), 5I2 mAb (2 $\mu$g/ml) or rabbit anti-mouse Crry (antiserum, 1/1000) and developed using peroxidase conjugated second antibody and enhanced chemiluminescence detection reagents (ECL.Amersham International, Amersham, UK). As standards to enable estimation of levels of expression of CD59 and 5I2 Ag by astrocytes, these membrane inhibitors were purified from rat erythrocyte ghosts and similarly electrophoresed and blotted. Briefly, rat erythrocyte ghosts were extracted in 1% Triton-X-100 and subjected to sequential affinity chromatography on a 5I2 mAb : Sepharose 4B column and a TH9 mAb : Sepharose 4B column. Subsequent gel filtration of eluates containing 5I2 Ag or rat CD59 on a Superose 12 column (FPLC, Pharmacia, Milton Keynes, UK) gave a suitable standard for each inhibitor. Molecular weight (MW) determination and quantification were performed by gel scanning densitometry (UVP, Cambridge, UK).

*RNA preparation and reverse transcription–polymerase chain reaction (RT–PCR)*

Total RNA was isolated from five confluent flasks of astrocytes in passage 2 by acid guanidium thiocyanate–phenol–chloroform extraction.[37] RT–PCR was performed according to the protocol of Kawasaki,[38] using 1 $\mu$g total RNA, primer pd(N)$_6$ (Pharmacia) and MMLV reverse transcriptase (Gibco, Paisley, UK) for the RT reaction and specific 20-mer oligonucleotide primers and *Taq* DNA polymerase (Bioline, Finchley, London, UK) for 40 cycles of PCR in a Hybaid Omnigene Thermocycler (Hybaid, Teddington, UK). Rat CD59 specific primers used to amplify bases 123–426 were 5′-CTGCTTCTGGCTGTCCTCTG-3′ and 5′-ACGCTGTCTT-CCCCAATAGG-3′ giving an expected product size of 303 bp.[23] 5I2 Ag-specific primers used to amplify bases 99–432 were 5′-TGCTGCTGTTCTTGTCGCCA-3′ and 5′-AGGAGGAAC-CAATGAGGCGG-3′ giving an expected product size of 333 bp (EMBL access code L36532). PCR products were analysed by electrophoresis on 1·2% agarose gels containing ethidium bromide for ultraviolet detection. Further confirmation of amplification of specific cDNA was obtained by purifying amplified cDNA using a QIAquick PCR purification kit (QIAgen, Hybaid, Teddington, UK), automated sequencing (Applied Biosystems, Warrington, UK) and comparison of sequences by eye.

*Functional studies*

Rat astrocytes in passage 1 or 2 were washed twice in PBS and resuspended at $10^6$ cells/ml in the same buffer alone or containing 0·5 mg/ml sheep anti-rat 5′-nucleotidase and/or 0·1 mg/ml TH9 mAb and/or 0·1 mg/ml 5I2 mAb. These two mAb are both of the IgG1 subclass and do not themselves fix C. The anti-rat 5′-nucleotidase bound to rat astrocytes and efficiently activated the CP. After 45 min sensitization with antibody on ice, cells were washed twice and resuspended at $10^6$ cells /ml in GVB prior to incubation with varying doses of normal human serum (NHS) or normal rat serum (NRS) for 60 min at 37°. Subsequent addition of 2 $\mu$g/ml propidium iodide (PI) and FACScan analysis allowed quantification of PI-positive (dead) and PI-negative (live) cells for each cell treatment.

## RESULTS

### Rat astrocytes constitutively express CD59 and 5I2 Ag *in vitro*

Flow cytometric analysis of rat astrocytes demonstrated positive staining with TH9, 6D1 and 5I2 mAb and polyclonal anti-mouse Crry (Fig. 1a, b). Of the two anti-rat CD59 mAb tested, staining with TH9 was stronger than with 6D1. Staining with 5I2 mAb was comparable with that of TH9 mAb. Rat astrocytes plated out and grown in different FCS concentrations (1%, 2·5%, 5%, 10%) showed almost identical levels of expression of the regulatory proteins. For CD59, the mean fluorescence level for cells grown in 1% FCS was 69·8 (SD of quadruplicate measurements, 5·3), in 2·5% FCS, 67·6 (SD 1·3) and in 5% FCS, 66·7 (SD 3·4). Culture in 0% FCS caused rapid death of the cells. Expression levels were consistently higher on cells in passage 1 and slowly fell with increasing numbers of passages.

Double immunofluorescent staining of cells for GFAP and rat CD59 or 5I2 Ag confirmed cell surface expression of these inhibitors by the majority of rat astrocytes. Figure 2a,b shows cells stained brightly for GFAP also displaying a strong, granular staining pattern for rat CD59 over the entire cell surface. Using 5I2 mAb a similar but weaker staining pattern was observed (data not shown). We obtained stronger staining for 5I2 Ag on cells using polyclonal anti-mouse Crry (which is known to cross-react with 5I2 Ag, see Western blotting results

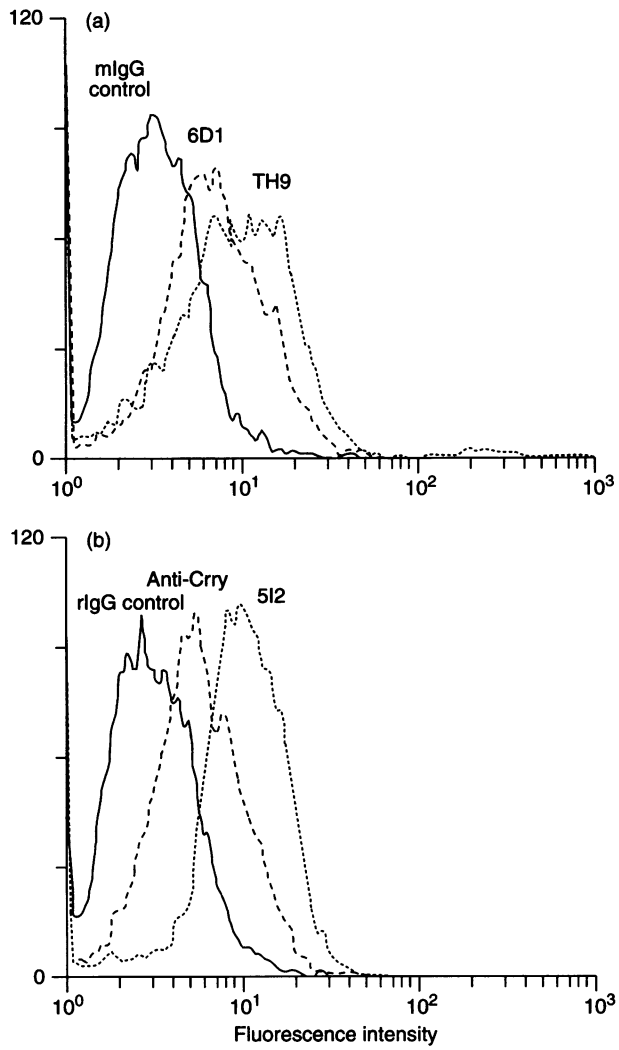


**Figure 1.** Flow cytomteric analysis of rat astrocytes using anti-rat CD59 mAb TH9 and 6D1 (a) and 5I2 mAb and rabbit anti-mouse Crry, both recognizing 5I2 Ag (b). Background staining with mouse IgG or rabbit IgG and the appropriate FITC-conjugated second antibody gave identical results. The *y* axis represents cell number.

and discussion) and these results, together with staining for anti-GFAP mAb are shown in Fig. 2c–f. Intense, particulate staining with anti-mouse Crry was apparent over the entire cell surface. The anti-mouse Crry-positive, GFAP-negative cells in Fig. 2e,f are likely to be fibroblasts on the basis of their morphology. Background staining using mouse or rabbit IgG or conjugated antibodies alone was low in each case.

### Confirmation of identity of rat CD59 and 5I2 Ag by SDS–PAGE/Western blotting and effect of PMA/cytokine stimulation

Astrocytes harvested from culture 24 hr post stimulation with cytokines or PMA all showed viability > 85%. Approximately $4 \times 10^6$ cells/confluent flask were harvested from stimulated or non-stimulated cells in passage 1 or 2. SDS–PAGE and Western blotting of cell lysates from non-stimulated astrocytes in passage 1 revealed a band at MW 20 000 detected by TH9 mAb (Fig. 3a, Co) comigrating with purified rat erythrocyte CD59 (Fig. 3a, 1–3) and bands at approximately 58 000 and 64 000 MW detected by 5I2 mAb (Fig. 3b, Co) comigrating with purified rat erythrocyte 5I2 Ag (Fig. 3b, 1–3). The MW of all bands were assessed by scanning densitometry in comparison to MW standards (New England Biolabs, Hitchin, UK). This data confirms expression of CD59 and 5I2 Ag by rat astrocytes. Development of similar blots with second antibody only identified the high MW bands in the standards as contaminating mouse IgG assumed to have leached from the immunoaffinity columns during purification. Using standards of known concentration and gel scanning densitometry we were able to estimate the level of expression of each protein per cell. Assuming homogeneous expression and 100% recovery of these proteins in the detergent lysates, rat astrocytes in passage 1 expressed 24 ng CD59/$10^6$ cells ($720 \times 10^3$ molecules/cell) and 62·5 ng 5I2 Ag/$10^6$ cells ($625 \times 10^3$ molecules/cell). No consistent change in level of expression of either protein was observed on stimulation for 24 hr with PMA or any of the cytokines tested as exemplified in Fig. 3a and b. Expression of both CD59 and 5I2 Ag decreased with increasing passage number as exemplified in Fig. 3a. Concentrated supernatants from the same cultures were similarly subjected to SDS–PAGE and Western blotting but failed to reveal the presence of soluble forms of CD59 or 5I2 Ag under any circumstances (data not included). Western blotting of astrocyte lysates revealed that the polyclonal anti-mouse Crry antibody recognized bands at 58 000 and 64 000 MW, identical to those recognized by 5I2 Ag mAb (data not shown).

### Expression of rat CD59 and 5I2 Ag mRNA

Using rat CD59- and 5I2 Ag-specific oligonucleotide primers we investigated the presence of mRNA for each of these proteins in rat astrocytes by PCR. The predicted 303 bp product was amplified from rat astrocyte cDNA using CD59 specific-primers (Fig. 4, lane 1) and the predicted 333 bp product using 5I2 Ag-specific primers (Fig. 4, lane 2). Controls using single primers revealed no specific bands. Purification of the specific amplified products and automated sequencing revealed > 90% identity over 90 bp with the published sequence for rat CD59 and 100% homology over 207 bp with the published sequence for 5I2 Ag. Errors introduced by the *Taq* polymerase probably account for the few mismatches with the published sequence for rat CD59.

### Rat CD59 and 5I2 Ag restrict CP-mediated lysis of rat astrocytes

Rat astrocytes were resistant to homologous C activation in the absence of C-fixing antibody and blocking antibodies to CD59 and/or 5I2 Ag failed to increase lysis of these cells (Fig. 5a). Background lysis in the absence of serum was high (up to 45%), reflecting the fragility of these cells, and was subtracted in all experiments. CP activation of homologous serum using polyclonal anti-rat 5′-nucleotidase caused killing of rat astrocytes and blocking antibodies to CD59 and 5I2 Ag enhanced the resulting cytotoxicity (Fig. 5b). The 5I2 mAb alone increased the susceptibility of these cells to homologous complement whereas anti-rat CD59 alone induced no enhancement of lysis. However, in combination with 5I2 mAb, anti-rat

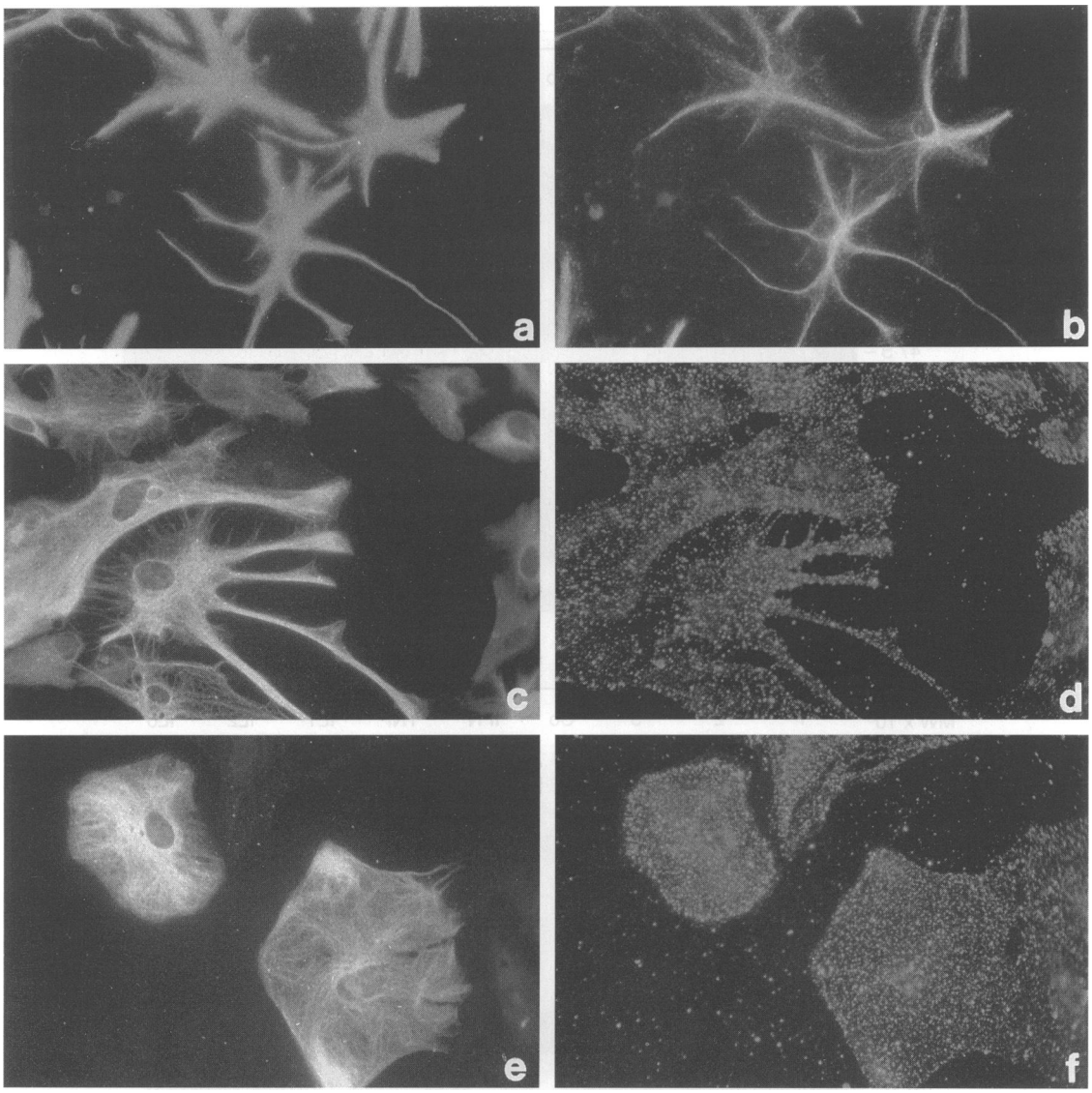


**Figure 2.** Double immunofluorescent staining of rat astrocytes. a, b: Cells double-stained with polyclonal anti-GFAP/rhodamine-conjugated second antibody and 6D1 mAb/FITC-conjugated second antibody (a; rhodamine optics. b; fluorescein optics). c, d and e, f: cells double-stained with anti-GFAP mAb/FITC-conjugated second antibody and polyclonal anti-mouse Crry/rhodamine-conjugated second antibody (c, e; fluorescein optics. d, f; rhodamine optics).

CD59 induced enhancement of lysis greater than that seen with 5I2 mAb alone. Killing of rat astrocytes by NHS alone shown in Fig. 5c was assumed to be due to CP activation by natural antibody in the NHS and was enhanced by the CD59 and 5I2 Ag-blocking antibodies. These data, together with those in Fig. 5d, where polyclonal anti-rat 5′ nucleotidase was used as sensitizing antibody to enhance CP activation on astrocytes, demonstrate an effect of the blocking antibodies on astrocyte killing in heterologous serum similar to that described above for homologous serum. Indeed, blocking rat CD59 induced a greater enhancement of lysis when the cells were exposed to heterologous rather than homologous C.

## DISCUSSION

In this paper we have investigated rat astrocyte expression of intrinsic membrane proteins responsible for the regulation of C activation on homologous cells. The presence of CD59 and 5I2 Ag were demonstrated by indirect immunofluorescent staining and flow cytometric analysis or fluorescence microscopy, SDS–PAGE/Western blotting and RT–PCR analysis. Rat astrocyte CD59 had a MWof 20 000, almost identical to that of rat erythrocyte CD59 (21 000).[22] The MW of rat astrocyte 5I2 Ag was 58 000 and 64 000, identical to that of rat erythrocyte 5I2 Ag in our hands but differing slightly to the published MW of 55 000 and 65 000.[18] Polyclonal anti-mouse Crry showed strong cross-reactivity with bands at 58 000 and 64 000, comigrating with purified rat 5I2 Ag (data not shown). This is not suprising given the high degree of homology between mouse Crry and 5I2 Ag.[19] Thus, the data from indirect immunofluorescent studies using anti-mouse Crry served to confirm expression of 5I2 Ag indicated by staining with 5I2 mAb.

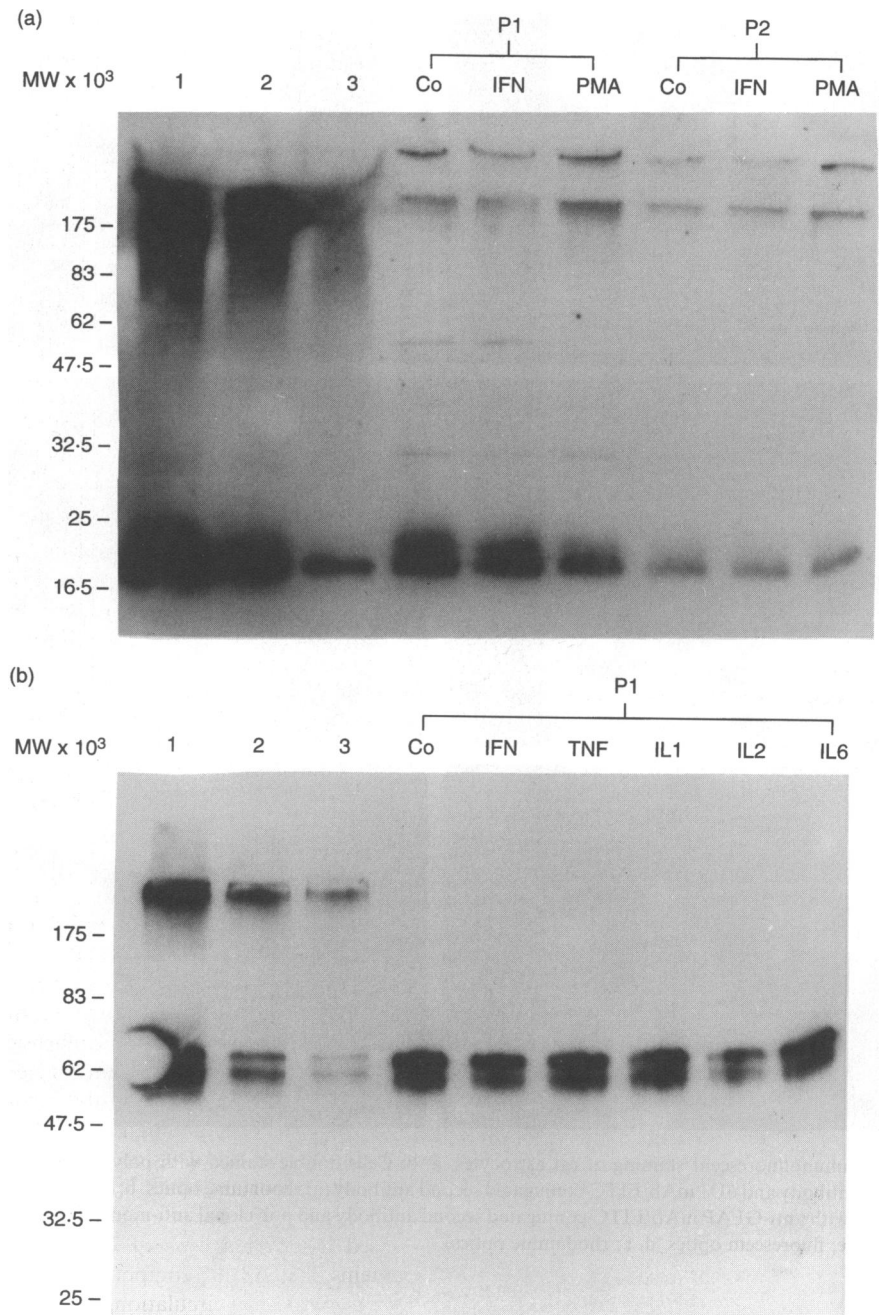


**Figure 3.** Western blotting of rat astrocyte lysates for CD59 and 5I2 Ag. Cell lysates from astrocytes in passage 1 or 2 (P1 or P2) unstimulated or stimulated with IFN-$\gamma$, TNF-$\alpha$, IL-1$\beta$, IL-2, IL-6 or PMA as indicated were subjected to SDS–PAGE and Western blotting using TH9 mAb (A. 5 $\mu$g/ml) or 5I2 mAb (B. 2 $\mu$g/ml) as primary antibodies. Purified proteins of known concentrations served as standards for quantitation: 8, 4 and 2 ng rat CD59 (a, lanes 1–3) and 16, 8 and 4 ng/ml 5I2 Ag (b, lanes 1–3). MW markers are indicated to the left (MW $\times 10^{-3}$).

This *in vitro* evidence for expression of CD59 and 5I2 Ag by astrocytes confirms and extends previous data describing expression of these C regulators by rat and human glial cells and the effects of cytokine stimulation on this expression. Rat astrocyte expression of CD59 protein and unidentified proteins that cross-react with polyclonal antibodies to human DAF and human MCP has been demonstrated[7,32] and human astrocytes have been shown to express CD59, DAF and MCP[28–31] but not CR1.[29] Expression of MCP and CD59 by human astrocytes was unaffected by stimulation with IFN-$\gamma$, IL-1$\beta$, IL-6, TNF-$\alpha$ or PMA (ref. 29 and our unpublished observation) and DAF expression was unaffected by IL-1$\beta$, IL-6 or TNF-$\alpha$, but up-regulated by IFN-$\gamma$ or PMA (our unpublished observation). In contrast to these results obtained using cultured cells, immunohistological investigation of CD59 and 5I2 Ag expression in the CNS revealed no apparent expression of these proteins in rat brain[25] and a similar apparent lack of expression or low expression in human

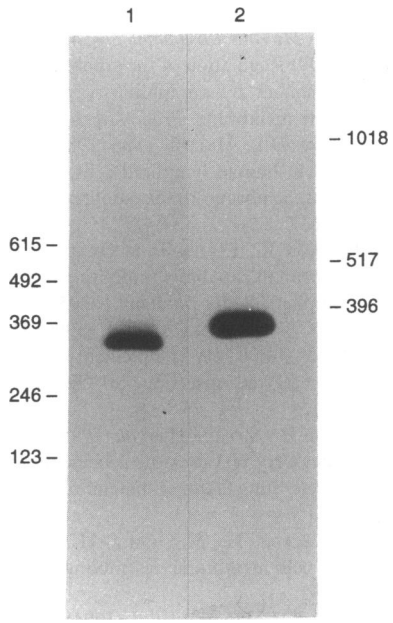


**Figure 4.** RT–PCR analsis of rat CD59 and 5I2 Ag. Amplification of rat astrocyte cDNA using rat CD59-specific primers (lane 1) and 5I2 Ag-specific primers (lane 3). Ethidium bromide staining of agarose gel (1·2%). DNA size markers in bp are indicated on either side.

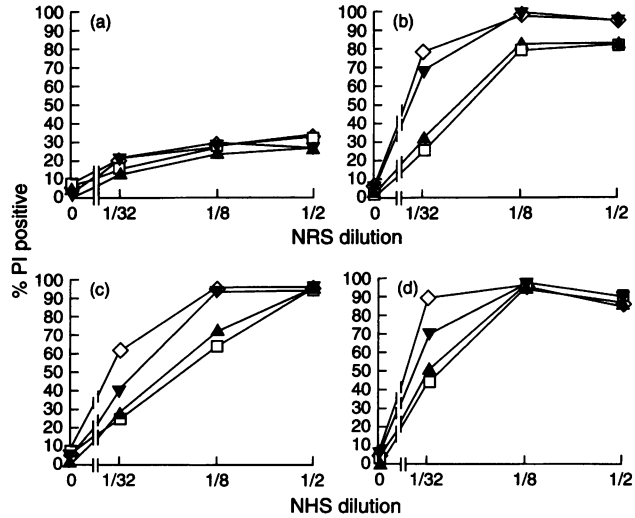


**Figure 5.** Effect of TH9 and 5I2 mAb on C-mediated lysis of rat astrocytes. Cells were preincubated with 5I2 mAb (▼), TH9 mAb (▲) or both (◇) or neither (□). a, b: Incubation with various dilutions of NRS in the absence (a) or presence (b) of C-fixing antibody (sheep anti-rat 5′-nucleotidase). c, d: Incubation with various dilutions of NHS in the absence (c) or presence (d) of C-fixing antibody (sheep anti-rat 5′-nucleotidase). Cell viability was assessed by uptake of PI and FACScan analysis. Background lysis in the absence of serum was subtracted from each value. Results are representative of three separate experiments. SD are omitted for clarity but were always < 10% of the value.

brain.[27,28] Whether these differences represent problems of sensitivity is not certain but our findings that cultured cells express similar levels of these proteins when cultured in different amounts of serum and that the highest levels of expression are found on cells in passage 1 make it unlikely that the results reported here are artefactual. Also we have for the first time confirmed expression of these proteins by rat astrocytes at the mRNA level.

The functional significance of CD59 and 5I2 Ag in protection of rat astrocytes from C-mediated lysis was shown by the ability of blocking mAb to enhance lysis of astrocytes by NHS or NRS when C is activated by the CP. Rat astrocytes demonstrated little spontaneous activation of the CP (Fig. 5a) although some additional cell death did occur on prolonged incubation at 37°. Polyclonal anti-rat 5′-nucleotidase activated the CP in homologous serum and efficiently induced killing of rat astrocytes (Fig. 5b). In NHS spontaneous activation of the CP, probably by natural antibody, occurred and was enhanced by sensitization of astrocytes with polyclonal anti-rat 5′-nucleotidase (Fig. 5c and d). Killing caused by CP activation in either homologous or heterologous serum was enhanced by mAb blocking the function of rat CD59 or 5I2 Ag. Our data indicate that 5I2 antigen is the primary membrane protein responsible for rat astrocyte resistance to CP activation. CD59 contributes little to protecting astrocytes when the function of 5I2 Ag is intact. However, when the function of 5I2 Ag is blocked by antibody, the inhibitory effects of CD59 on C5b-9 are revealed. Our data demonstrating no spontaneous activation of homologous C by type 1 astrocytes when CD59 is blocked by mAb contrast with findings from type 2 astrocytes (identified on morphological grounds) which spontaneously activated homologous C and where blocking of CD59 with mAb rendered the cells susceptible to killing in the absence of sensitizing antibody.[7]

Although it was not the intention of this investigation to examine expression of CD59 and 5I2 Ag by rat oligodendrocytes, some of the cultures did contain occasional contaminating oligodendrocytes and indirect immunofluorescent staining of these cells with anti-galactocerebroside and 6D1 or 5I2 mAb demonstrated that rat oligodendrocytes did not express CD59 (as shown previously)[7] but did express 5I2 Ag (a novel observation). This preliminary finding is being pursued in pure cultures of rat oligodendrocytes.

It is important for CNS cells to be protected from homologous C because blood–brain barrier breakdown caused by inflammation or infection allows entry of plasma proteins, including C components, into the CNS. C activation products from the circulation or generated within the CNS are potentially damaging to glial cells. Evidence for the *in vivo* generation of C fragments in the CNS has been obtained in human diseases and animal models[2–4] and C has been implicated as a pro-inflammatory and myelin-damaging factor in the pathogenesis of demyelinating and degenerative CNS disease.[1] The source of C for the perpetuation of these pathologies had been assumed to be the blood, thus requiring a leaky blood–brain barrier. However, recent evidence has clearly demonstrated that intrathecal biosynthesis of functionally active C components occurs in both humans and rats. Human astrocyte-derived cell lines synthesize and secrete all components of the classical, alternative and terminal pathways of C.[31,39,40] Rat astrocytes constitutively express C3, factor B (mRNA and protein), C4 binding protein and factor H (mRNA level only) and expression of C3 is enhanced by inflammatory stimuli and viral infection.[41,42]

Thus, in the human and perhaps also the rat CNS there is the potential to generate a complete C system intrathecally which is up-regulated by inflammatory cytokines. The presence of membrane C inhibitors (CD59 and 5I2 Ag in the rat) on astrocytes, which we suggest are the predominant C-expressing cell in the CNS, is therefore important to the survival of these cells and the relative or absolute deficiency of C inhibitors on other CNS cells may be a contributor to pathology. Oligodendrocytes and a subpopulation of astrocytes putatively identified as Type 2 (but not Type 1 astrocytes as here demonstrated) spontaneously activate homologous C via the CP.[7] Although activation of C on these cells *in vivo* is potentially damaging, we propose that it may also have a physiological role. Spontaneous, low-level activation of intrathecally produced C on oligodendrocytes and susceptible astrocytes (and perhaps also on neurons) may have non-lethal activating effects which are important to normal functioning of glia.

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