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Complement regulatory protein expression by a human oligodendrocyte cell line: cytokine regulation and comparison with astrocytes

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SUMMARY

Rat oligodendrocytes spontaneously activate complement (C) and lack the C inhibitor CD59. As a consequence, rat oligodendrocytes are susceptible to lysis by autologous C *in vitro*. Expression of C inhibitors on human oligodendrocytes *in vitro* and other human glia has yet to be well characterized. We have previously shown expression at the mRNA level of the membrane inhibitors CD59, decay-accelerating factor (DAF; CD55) and membrane cofactor protein (MCP; CD46) in human astrocytes. We here examine the expression of membrane and secreted C inhibitors by the oligodendrocyte cell line, HOG. HOG cells abundantly expressed CD59, assessed at protein and mRNA level, and expressed DAF and MCP, albeit at a lower level. Expression of all three inhibitors was enhanced by incubation with interferon-γ or with phorbol ester (PMA). Complement receptor type 1 (CR1; CD35) was neither expressed constitutively nor induced by cytokines. HOG also constitutively secreted C1-inhibitor, S-protein and clusterin. Factor H was secreted only after stimulation with cytokines. C4b binding protein was expressed at a very low level and was detected only at the mRNA level by reverse transcriptase–polymerase chain reaction (RT–PCR). For comparison, astrocyte expression of CD59, DAF, MCP and CR1 was confirmed at the mRNA and protein levels. HOG did not activate C spontaneously, as judged by the lack of deposition of C fragments, and were not lysed by C even after inhibition of CD59 and DAF using specific monoclonal antibodies.

INTRODUCTION

The complement (C) system is an important pro-inflammatory component of the immune system with the capacity to recognize and eliminate a large variety of pathogens or abnormal cells. C is made up of more than 25 plasma and membrane proteins arranged in two activation cascades (alternative and classical pathway) and a lytic pathway (with the genesis of a membrane attack complex, MAC).[1] C fragments generated during activation can attract and activate phagocytic cells and opsonize foreign or damaged cells for elimination by phagocytosis.[2] Each of the C pathways is very tightly regulated *in vivo* by complement regulatory proteins. C1-inhibitor (C1-INH), C4b binding protein (C4bp), factor H (FH), factor I (FI), S-protein and clusterin are soluble C inhibitors present in the plasma and produced mainly by the liver but also by other cell types in the tissues.[3] Membrane cofactor protein (MCP, CD46), decay-accelerating factor (DAF, CD55), complement receptor type 1 (CR1, CD35), and CD59 are membrane-bound C regulatory proteins expressed by most nucleated cells in order to control C activation on self cells.[1] We are interested in the role of C in the normal brain and in central nervous system (CNS) inflammation. Evidence implicating C in demyelination, neurodegeneration and other CNS pathologies has accumulated during the last few years, although the relative importance of C remains a subject of debate.[4] Cells in the brain are normally sheltered from C in the plasma and appear, at least *in vitro*, to be particularly susceptible to C-mediated injury.[5–7] C levels in the CNS may increase as a consequence of blood–brain barrier leakage following injury or inflammation and may exacerbate injury by damaging C susceptible cells such as neurons and oligodendrocytes. Alternatively, complement may be synthesized intrathecally. We have recently shown that CNS astrocytes *in vitro* have the capacity, under appropriate cytokine drive, to express a complete, functional C system,[8–10] raising the possibility that astrocyte-derived C might damage surrounding neurons and oligodendrocytes, and contribute to pathology in inflammatory CNS diseases.[1]

In the presence of human or rat serum, differentiated rat

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Abbreviations: C, complement; C4bp, C4b binding protein; C1-INH, C1-inhibitor; CNS, central nervous system; CR1, complement receptor type 1 (CD35); DAF, decay-accelerating factor (CD55); FH, factor H; GPI, glycosyl-phosphatidylinositol; HOG, human oligodendrocyte cell line; MAC, membrane attack complex; MCP, membrane cofactor protein (CD46); NHS, normal human serum; PIPLC, PI-specific phospholipase C.

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oligodendrocytes and, to a lesser, extent rat 0–2 A progenitors, activate directly the classical pathway of C,[5,6,11,12] probably by direct binding of C1q to myelin proteins.[13,14] In contrast, rat type I astrocytes, meningeal cells and Schwann cells are not sensitive to C.[5,12] Rat type II astrocytes activate directly the classical pathway of C but are resistant to lysis because of their capacity to express CD59, an inhibitor that blocks the formation of the MAC, and therefore protects from C cytotoxicity and cytolysis.[12] Rat oligodendrocytes do not express CD59 and are lysed by C.[11,12] Pure cultures of human oligodendrocytes are more difficult to obtain and little is known about their sensitivities to C.

In this study we have used a cell line that shares numerous characteristics with the human oligodendrocyte as a model to analyse complement activation and expression of complement regulatory proteins by oligodendrocytes *in vitro*. We here report that the human oligodendrocyte-derived cell line (HOG) is resistant to C and expresses *in vitro* CD59, DAF, MCP and the fluid phase inhibitors C1-INH, FH, S-protein, and clusterin. No expression of CR1 was observed even after cell stimulation with various agents. We further analysed the effects of cytokines on the expression of C inhibitors by HOG and compared this with the capacity of astrocytes and astrocyte cell lines to express the same C inhibitors.

MATERIALS AND METHODS

Chemicals, cytokines, antibodies and C components

Recombinant human interferon-γ (IFN-γ), interleukin-1β (IL-1β) and tumour necrosis factor-α (TNF-α) were the gift of Hoffman la Roche (Nutley, NJ). Recombinant human IL-2 and IL-6 were the gift of Dr C. Fagin (Department of Haematology, UWCM). Dexamethasone (DXM) was from Organon, Warwick, UK. PMA, leupeptin, pepstatin, monoclonal anti-glial fibrillary acidic protein (GFAP) and monoclonal anti-galactocerebroside (GC) were from Sigma (Poole, Dorset, UK). Monoclonal antibodies: anti-C1 inhibitor (anti-C1-INH) was the gift of Dr Kwok Khan (Tenovus Institute, Southampton, UK) and anti-S protein (clone S142) were from this laboratory; anti-factor H (clone OX24) was the kind gift of Dr R. B. Sim (MRC Unit, University of Oxford, UK), anti-CR1 (clone mAb 543; access no. HB8592) was from the ATCC (American Type Culture Collection, Rockford, MD); anti-human clusterin (clones G7, E5) were the gift of Dr B. F. Murphy (St Vincent's Hospital, Melbourne, Australia); anti-human C4 bp were the gift of Dr A. Ischenko (IHPB, St Petersburg, Russia). Antibodies against CD antigens: CD35, CD11a, CD11b, CD11c, CD68, and anti HLA Dr were from Dako Ltd. (High Wycombe, Bucks, UK); CD55 (BRIC110 and BRIC216) and CD59 (BRIC229) were from IBGRL (Elstree, Herts, UK); CD46 (clones GB24 and Tra 2·10), were the gift of Dr J. P. Atkinson (St Louis, MO). Polyclonal antibodies (anti-C1-INH, anti-FH, anti-S protein, anti-C3c) were prepared in-house by immunization of rabbits using standard techniques. Immunoglobulin G (IgG) was purified by chromatography on protein A sepharose (Pharmacia, St. Albans, Herts, UK). Polyclonal anti-clusterin and anti-CD46 were the gifts of Drs Murphy and Atkinson, respectively. C components and C regulatory proteins (MCP, DAF, CD59) were prepared in the laboratory by affinity chromatography. Recombinant soluble CR1 and monoclonal anti-CR1 (clones 4D2, 3E10 and 3H7) were the gift of SmithKline Beecham (Harlow, Essex, UK). All reagents were thoroughly tested for purity and functional activity.

Cell preparation and culture

The HOG was generously provided by Professor Glyn Dawson (Department of Biochemistry, University of Chicago, IL).

The phenotype of this cell has been characterized in detail and reported previously.[15] This cell line constitutively expresses various mRNAs for oligodendrocyte-specific proteins (2',3'-cyclic nucleotide 3' phosphodiesterase (CNPase), myelin basic protein (MBP), galactocerebroside, vimentin, and K7 keratin) but not astrocyte-specific proteins (GFAP).[16,17] We routinely checked the expression of galactocerebroside by HOG on cells in culture passage 20–40 in our laboratory. Primary cultures of human fetal astrocytes were obtained from Drs M. Tardieu and C. Hery (Kremlin-Bicètre, Paris, France) or were grown in-house from fetal brain supplied by the MRC Fetal Tissue Bank (London, UK). All cultures were established from fetal brain (6–10-week-old fetus) as described previously.[18,19] Experiments were conducted from passages 6–9 (day 30–60) where cultures contained > 95% GFAP positive cells and < 5% neurons, fibroblasts and microglia.

The four human astrocyte cell lines T98G, U118 MG, CB109, and CB193 ($GFAP^+$) were cultured and characterized as described previously.[8] Human monocyte-derived cell line THP1 was from the ATCC. Human erythroleukemia cell line K562 was obtained from European Collection of Animal Cell Cultures (ECACC, Salisbury, UK).

Cell phenotype was confirmed using either immunocytochemistry or by FACS analysis. Fetal astrocytes and astrocyte cell lines presented the phenotype ($GFAP^+$, $CD11b^-$, $CD35^{+/low}$, $HLADr^-$). Cells were stimulated with different concentrations of cytokines (20–1000 IU/ml) or with PMA (10^{-6} M) for various periods (24 hr to 72 hr).

Monocyte cell line THP1 was used as a control and was characterized as monocyte/macrophage (the latter after PMA treatment for 3 days) cell phenotype with, respectively, for monocyte/macrophage: $GFAP^{-/-}$; $CD11a/b/c^{+/+\ +\ +}$; $CD68^{+/+\ +\ +}$; $CD35^{+/+\ +}$; $HLADr^{+/+\ +}$.

Immunocytochemistry

For staining, cells were cultured on sterile glass coverslips for 5–8 days. After washing with phosphate-buffered saline (PBS), cells were fixed with 1% formaldehyde for 20 min and then were washed intensively in PBS/0·2 M glycine. For GFAP immunostaining cells were fixed and permeabilized with a mixture of 95% ethanol/5% acetic acid for 5 min at −20°. Antibodies were used at optimal dilution [1 μg/ml in PBS/1% bovine serum albumin (BSA)] and incubated with fixed cells for 30 min at 37° in a humid chamber. After washing, coverslips were incubated for 30 min at 37° with fluorescein isothiocynate (FITC)-labelled secondary antibody $F(ab')2,^{1/100}$ rabbit anti-mouse IgG (Dako) or goat anti-rabbit IgG (Sera-Lab, Sussex, UK). After intensive washing, coverslips were mounted in Citifluor (Citifluor Ltd, London, UK) and sealed. Fluorescence was imaged by confocal laser scanning microscopy on a Leica TCS microscope (Leica UK Ltd, Milton Keynes, UK). Twelve optical sections were collected per field at 0·3–0·5 μm intervals from the bottom to the top of the cell. Sections were then either assembled as extended focus views or individual sections were viewed as a gallery.

Flow cytometry

HOG, fetal astrocytes or cell lines were harvested from culture by incubation in FACS buffer (PBS containing 2% bovine serum albumin and 0·1% NaN_3) supplemented with 10 mM EDTA. Cells were washed and resuspended at 10^6 cells/ml in the same buffer without EDTA, incubated with the appropriate primary antibody (0·5–5 μg/ml unless stated otherwise) for 30 min on ice, washed three times in cold FACS buffer, incubated with the appropriate R phycoerythrin-labelled secondary antibody (Dako; diluted 1/100) for 30 min on ice and washed a further three times prior to analysis on a Becton-Dickinson FACScan (San Jose, CA). In some experiments cells in suspension were treated with PI-specific phospholipase C (PIPLC; Peninsula Labs, St Helens, UK) before FACS analysis. HOG and K562 (10^6 cells in phosphate buffer) were incubated with 0·4 IU/ml of PIPLC at 37° for 1 hr.

Cell lysates, concentrated cell culture supernatants and Western blotting

Western blotting was performed on unstimulated cells or cells stimulated with 200 IU/ml of IFN-γ or PMA (10^{-6} M) for 24 hr. Cell pellets (equivalent of 10^7 cells) were solubilized in 1 ml of PBS containing 1% Triton X-100 together with enzyme inhibitors as previously described.[8,9] Samples were fractionated on sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE), electroblotted onto nitrocellulose and stained with appropriate antibodies essentially as previously described.[8,9] Blots were developed using the Enhanced Chemiluminescence method (ECL, Amersham International, Amersham, UK). Prestained protein markers from Biorad and NBL (broad range) were used as a molecular weight standard. Blots were quantified using a UVP densitometry system (UVP Ltd, Cambridge, UK), calibration being achieved using pure proteins (CD59, DAF, MCP, CR1) or serum (clusterin, S-protein, C1-inhibitor, factor H, C3). For quantification in all experiments, cells were removed by gentle scraping or by brief trypsinization (0·1%), counted, and results were expressed as nanograms of C protein/10^6 cells. Human monocyte cell line (THP1) was used as positive control.

For western blotting of secreted proteins, 24–72 hr cell supernatants from unstimulated or cytokine-stimulated cells incubated in the absence of fetal calf serum (FCS) were harvested, dialysed overnight against buffer (50 mM Tris, 10 mM EDTA, 0·1 mM PMSF) and concentrated × 10 by lyophilization prior to fractionation on SDS–PAGE.

C attack

HOG cells were cultured in Lab Tek chamber slides (Gibco Life Technologies, Paisley, UK) or 96-well plates to near-confluence, washed and incubated at 37° for 30 min with dilutions of normal human serum (NHS) 1:2–1:64 in Veronal buffer (CFD, Oxoid, Hampshire, UK) containing 1% BSA. Cells were then either incubated with propidium iodide (PI, 0·5 μg/ml final concentration) to assess killing or immunostained for C fragments. Cell lines were also pre-incubated with non-C fixing neutralizing antibody against DAF (BRIC110 and BRIC216 each at 5 μg/ml) and/or CD59 (BRIC229, 10 μg/ml) (15 min) prior to incubation with NHS (30 min, 37°). We chose to measure killing by PI staining of adherent cells rather than of cells in suspension because all methods for removing adherent cells from the plastic caused a high percentage cell killing. Cell death was estimated by counting total and PI-positive cells in near-confluent fields viewed using a Nikon fluorescence microscope (Nikon UK Ltd, Kingston, Surrey, UK). At least four fields and 100 cells were counted for each determination. In some experiments, cells were incubated for 10 min with NHS containing EDTA or Mg^{++}/EGTA (each at 10 mM) to inhibit C activation.

Table 1. List of oligonucleotides used and results of RT–PCR

Protein	EMBL* No.	Primer positions†	cDNA pdt	HOG	T98G	Hu astr.
CD 35 (CR1)	Y00816	363–877	514 bp	–	+/–	+
CD 46 (MCP)	Y00651	301–720	399 bp	+	+	+
CD 55 (DAF)	M30142	1043–1520	360 bp	+	+	+
CD59	X15861	241–590	349 bp	+	+	+
C1-INH	M13203	51–401	350 bp	+	+	+
C4bp	X02865	311–690	379 bp	+/–	+/–	+/–
S-protein	X03168	712–1050	329 bp	+	+	+
Clusterin	M25915	271–670	399 bp	+	+	+
FH	X07525	233–572	339 bp	+	+	+
C3	K02765	311–978	667 bp	–	+	+
Actin-β	M10278	311–800	489 bp	+	+	+

*EMBL, European Molecular Biology Laboratory genebank. †Primer positions (upstream and downstream) are given according to their position (nucleotide number) in the cDNA sequence from 5′ to 3′. The length of all oligonucleotide primers was 20 bp. RT–PCR was performed according to the protocol described in the Materials and Methods, using total RNA from unstimulated cells: HOG, human oligodendrocyte cell line; T98G, astrocyte cell line; Hu astr., Human fetal astrocyte, culture passage 7. The results are given according to the quantity of each RT–PCR cDNA product detected after ethidium bromide staining. (–, not detectable; –/+, faint and reproducible signal; +, strong cDNA product).

RNA extraction and RT–PCR

Total RNA from cell lines was prepared using the Ultraspec RNA system according to the manufacturer instructions (Biotex lab. Houston, TX). Total RNA was prepared from primary culture of human astrocytes by the guanidium isothiocyanate procedure followed by centrifugation onto a CsCl cushion. RNA integrity was confirmed on agarose gels and concentrations were determined from absorbance at 260 nm.

Prior to PCR steps, the RT was carried out at 37° for 60 min in 30 μl (final volume) with 1 μg of total RNA, 60 U RNasin (Promega, Southampton, UK), dNTPs 1 mM, 250 pmol random hexamer primers (pdN6 from Pharmacia biotech limited, Herts, UK) and 500 U Moloney murine leukaemia virus (MMLV) reverse transcriptase (Gibco, Life Technologies, Paisley, UK) in the reaction buffer (10 mM Tris/HCl, 15 mM KCl, 0·6 mM $MgCl_2$, 5 mM DTT). The absence of contaminants was routinely checked by RT–PCR assays of negative control samples, in which the RNA samples were replaced with sterile water, or MMLV reverse transcriptase was not added.

PCR was performed in a Hybaid Omnigene (Teddington, UK) thermocycler. The reaction was carried out with 3 μl of reverse transcribed RNA mixture, in a 100 μl final reaction volume with 10 pmol of each oligonucleotide (see Table 1) (Bioprobe systems, Paris, France), in × 10 buffer (Biotaq buffer: 100 mM Tris–HCl, 50 mM KCl, 15 mM $MgCl_2$, 1% triton X-100) dNTP 200 μM and 1·25 U of BIOTaq DNA polymerase (Bioline UK Ltd, Finchley, UK). The PCR conditions used were: denaturation step at 95° for 5 min, five cycles (94° for 10 seconds, annealing at 60° for 60 seconds, extension at 72° for 120 with a ramping 6 seconds/°, from annealing to extension), 30 cycles as above without ramping, and elongation at 72° for 10 min.

All samples were subjected to RT–PCR for β-actin as a positive control and as an internal standard not affected by any cytokines or PMA treatment. Samples of RT–PCR products were loaded onto a 1·2% agarose gel (containing 50 μg ethidium bromide/200 ml gel) in 1 × TBE buffer and separated by electrophoresis at 120 V for 3–4 hr. RT–PCR products were visualized under UV light. DNA Ladders (123 bp and 1 kb) from Gibco were used as DNA size markers.

RESULTS

HOG cells express membrane C inhibitors (MCP, DAF, CD59) and do not spontaneously activate C *in vitro*.

We first observed the expression of MCP, DAF and CD59 by unstimulated and cytokine-stimulated HOG. By indirect immunofluorescence staining of adherent cells and confocal microscopy analysis we found that CD59 was abundantly

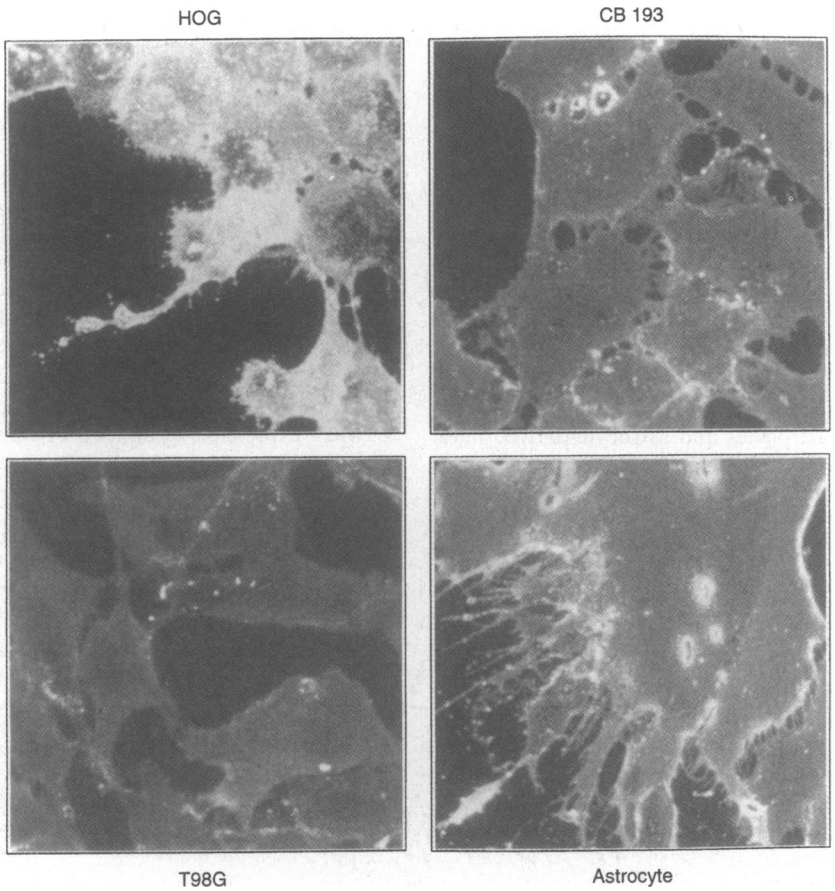

Figure 1. Immunolocalization of CD59 on human oligodendrocyte (HOG), fetal astrocyte, and astrocyte cell line (CB193 and T98G) by indirect immunofluorescence and confocal laser microscopy scanning. Cells were cultivated on glass coverslips, fixed with formaldehyde and processed for immunostaining using a monoclonal anti-CD59 (BRIC 229), followed by a FITC-conjugated anti-mouse immunoglobulins. (× 400).

Table 2. Expression of membrane regulatory proteins of complement on human glial cells. FACS analysis was carried out on HOG (human oligodendrocyte cell line), T98G (human astrocyte cell line) and on human fetal astrocyte (culture passage number 8) using anti-CR1 (4D2), anti-DAF (BRIC 216), anti-MCP (TRA 2.10) and anti-CD59 (BRIC 229). Co is the control staining obtained with an irrelevant antibody (anti-FH, OX24). Intensity of the staining is represented as the mean of fluorescence analysed on FL2 channel from 10 000 cells. The number of analysis (n) was (HOG, $n = 7$; T98G, $n = 10$; Hu. fetal astrocyte, $n = 2$)

	Mean of fluorescence (FL2)		
	HOG	T98G	Hu. astrocyte
Co	5·4	6	5·9
CR1 (CD35)	6·6	39·4	34·7
DAF (CD55)	76·3	361·4	87·8
MCP (CD46)	60·5	170·9	157·2
CD59	1064	1097	1388·3

expressed by HOG (Fig. 1). Fetal astrocytes and astrocyte cell lines (CB193 and T98G) also abundantly expressed CD59 (Fig. 1). CD59 was mainly expressed on the HOG cell membrane but in permeabilized cells some specific staining was also detected within the cell cytoplasm. Membrane staining on each cell type was granular in appearance. The same distribution pattern was also observed for MCP and DAF but the staining was less intense (not shown). CR1 was not detected on HOG and only a faint but reproducible staining was observed on human fetal astrocytes and astrocyte cell lines.

To confirm and to quantify the immunostaining, the same staining protocol was applied to cells in suspension and fluorescence detected by flow cytometry (Table 2). This method revealed abundant expression of CD59 by 100% of HOG and a much weaker expression of DAF and MCP. The level of CD59 expression by HOG was identical to the level of expression by astrocytes but expression of DAF and MCP was 2–3 times less as judged by mean fluorescence intensity (Table 2). CR1 was not detected on HOG but was clearly present at low level on astrocytes and astrocyte-derived lines (Table 2). All these levels of expression were stable over multiple passages in culture. Incubation with a panel of cytokines prior to FACS analysis revealed that only IFN-γ (200 IU/ml) upregulated MCP, DAF, and CD59 expression by HOG and fetal astrocyte (1·3–1·5-fold after 24 hr) but had no effect on expression by either astrocyte cell line.

PMA treatment caused a similar increase in expression of membrane inhibitors on HOG cells which peaked after 3 days of treatment when the cells had undergone a major change in morphology (long cell processes characteristic of differentiated cells).

Neither IFN-γ nor PMA induced the expression of CR1 by HOG.

To confirm that CD59 and DAF on HOG cells were glycosyl-phosphatidyl inositol (GPI) anchored we treated the cells with PI-specific phospholipase C (PIPLC).

PIPLC treatment caused the release of about 70% of both DAF and CD59 whereas expression of MCP, a transmembrane protein, was unaffected by this treatment (Table 3). Release of DAF and CD59 from the control erythroleukaemia cell line, K562, was 50% and 64%, respectively (Table 2). This cell line has previously been reported to have a GPI anchor that is inefficiently cleaved by PIPLC, perhaps explaining the inefficient cleavage observed here.[20] LFA-3 (CD58) is expressed by most nucleated cells in both GPI-anchored and transmembrane forms. PIPLC treatment caused release of only 30% of LFA-3 expressed by HOG indicating that on this cell type less than half of the LFA-3 was GPI anchored.

Expression of membrane C inhibitors (MCP, DAF, CD59) by HOG and astrocytes was also assessed by western blot analysis of cell lysates and of cell culture supernatants to detect the presence of soluble forms (Fig. 2).

CR1 was weakly detected in lysates of human fetal astrocytes and astrocyte cell lines (T98G, CB193, U118MG, but not CB109) with an apparent molecular weight (MW) identical to the MW (230 kDa) of purified recombinant CR1 and CR1 expressed by the cell control, THP1. No CR1 was detected in HOG cell lysates and cell culture supernatants even after stimulation with IFN-γ and PMA.

MCP was abundantly and constitutively expressed by HOG ($\sim$10–20 ng/10^6 cells), fetal astrocytes and all cell lines. Two bands were detected with a polyclonal anti-MCP, a characteristic of MCP on SDS–PAGE,[21] with an upper band (55–58 kDa) and a lower band (45–50 kDa) in unreduced conditions. Treatment with IFN-γ or PMA (24 hr) increased the level of MCP expression by a factor of 1·5 and 3 times, respectively (Fig. 2).

DAF was also constitutively expressed by HOG ($\sim$5–10 ng/10^6 cells), astrocytes and all cell lines, with the exception of the human glioblastoma CB109. DAF expressed by HOG

Table 3. Cleavage of GPI anchor CD55, CD59 and CD58 expressed on human oligodendrocyte cell line HOG and on control cell line K562. FACS analysis was carried out without or after treatment of cells with 0·4 IU/ml/10^6 cells of PIPLC for 1 hr at 37°. The same antibodies and protocol (mentioned in Table 2 legend) were used, and in addition we have used an anti-LFA-3 (CD58) (clone BRIC 5)

	Mean of fluorescence (FL2)			Mean of fluorescence (FL2)		
	HOG	HOG + PIPLC	% cleavage	K562	K562 + PIPLC	% cleavage
Co	4·8	4·7	–	2	2·1	–
DAF (CD55)	94·2	29·3	68·9	39·9	20·1	49·6
MCP (CD46)	33·2	37·8	–	54·8	50	–
CD59	1010	297·4	70·6	375·9	124·3	64·3
LFA-3 (CD58)	174·3	119·5	31·5	67·2	50·6	24·6

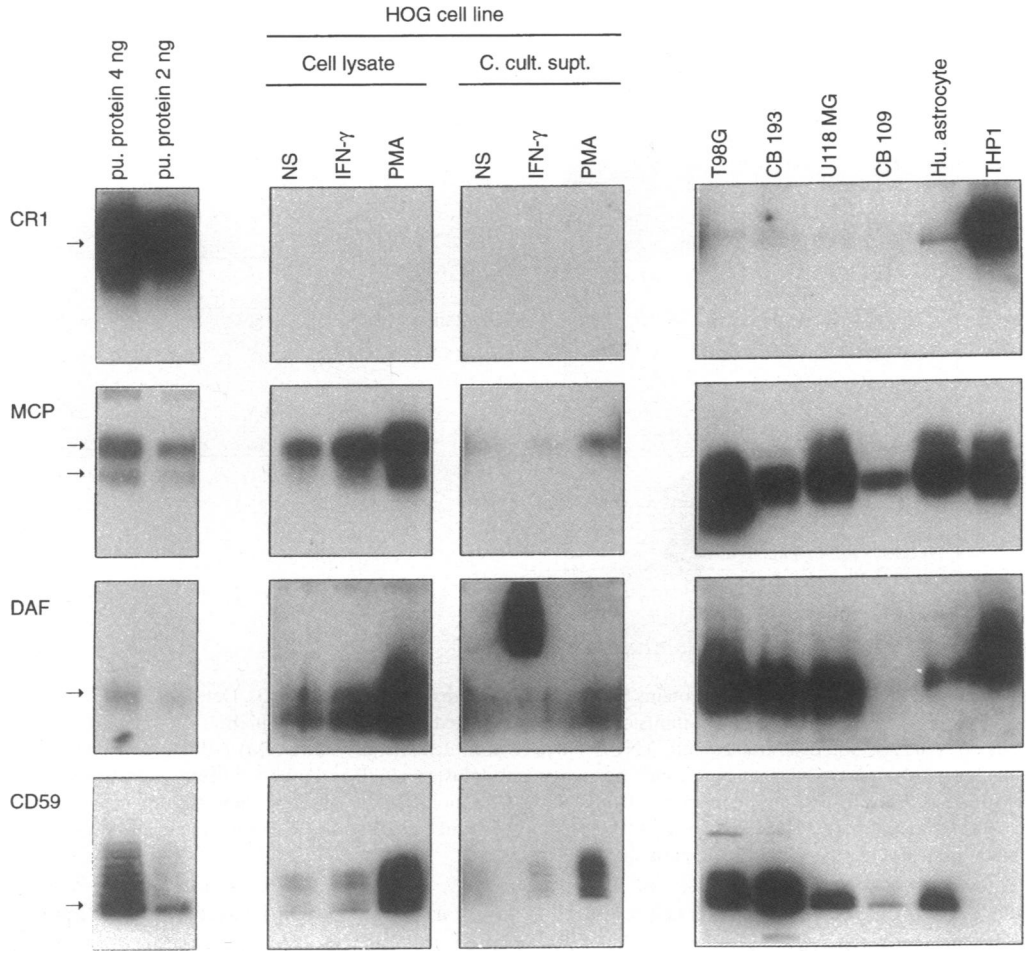

Figure 2. Expression of CR1, MCP, DAF and CD59 (membrane and soluble forms) by human glial cells: cell lysates and cell culture supernatants of human oligodendrocyte cell line (HOG), unstimulated (NS) or after 24-hr stimulation with IFN-γ (200 IU/ml) or PMA (10^{-6} M), and cell lysates from astrocyte cell lines (T98G, CB193, U118MG, CB109), fetal astrocyte and monocyte cell line (THP1) were analysed by western blot. Samples were analysed by SDS–PAGE (7·5% acrylamide, except for DAF, 10% and CD59, 15%) under non-reducing conditions, followed by western blotting and detection with polyclonal antibodies against CR1, MCP and DAF (0·2 μg/ml) and a monoclonal anti-CD59 (BRIC229, 0·5 μg/ml). Purified CR1, MCP, DAF, CD59 were used as controls to identify the correct molecular weight under non-reducing conditions (CR1, 230 kDa; MCP, 2 forms: 55/45 kDa; DAF, 60 kDa; CD59, 20 kDa) and to allow quantitation by densitometry, allowing all results to be expressed as nanograms of protein per 10^6 cells. THP1 was also used as a control. Cell loading per track was: 2×10^5 cells and cell culture supernatants, equivalent of 10^5 cells.

had an apparent MW identical to the 60 kDa purified erythrocyte DAF, analysed in parallel.[22]

IFN-γ and PMA increased the level of DAF expression by a factor of 2 and 3·5 times, respectively (Fig. 2). CD59 was constitutively expressed by HOG (~2–5 ng/10^6 cells), astrocytes and all astrocyte cell lines (~10–20 ng/10^6 cells) at an apparent molecular weight identical to that of purified erythrocyte CD59 (~20 kDa). The THP1 monocyte cell line was CD59-negative. IFN-γ had no significant effect on CD59 expression by HOG whereas PMA increased CD59 expression fourfold (Fig. 2).

Analysis of × 10 concentrated cell culture supernatants of HOG revealed the presence of soluble forms of MCP, DAF and CD59 (Fig. 2). PMA increased the expression of each of the soluble forms of the membrane inhibitors.

In order to examine C susceptibility, HOG cells were incubated for 1 hr at 37° with increasing dilution of NHS as a source of C and cell killing was measured using PI staining. At a 1:2 dilution of NHS less than 5% of adherent HOG were PI positive (data not shown). The absence of lysis was correlated with the low levels of C components opsonized on HOG membranes in the presence of NHS. By indirect immunofluorescence using monospecific polyclonal antibody, weak staining was obtained only for C3 whereas the staining for other C components (C1q, C4, C5, C8 and C9) was completely negative. Additionally, no staining was obtained with a monoclonal anti-MAC (B7) recognizing a neoepitope in the MAC. Neutralizing monoclonal antibodies against DAF and CD59 (BRIC 216 and BRIC 229, respectively) which efficiently inhibit the function of these inhibitors, neither enhanced killing of HOG by NHS nor opsonization of HOG with C fragments.

HOG express fluid-phase C inhibitors

Expression of the fluid-phase C inhibitors C1-INH, FH, S-protein and clusterin was assessed by immunostaining of fixed

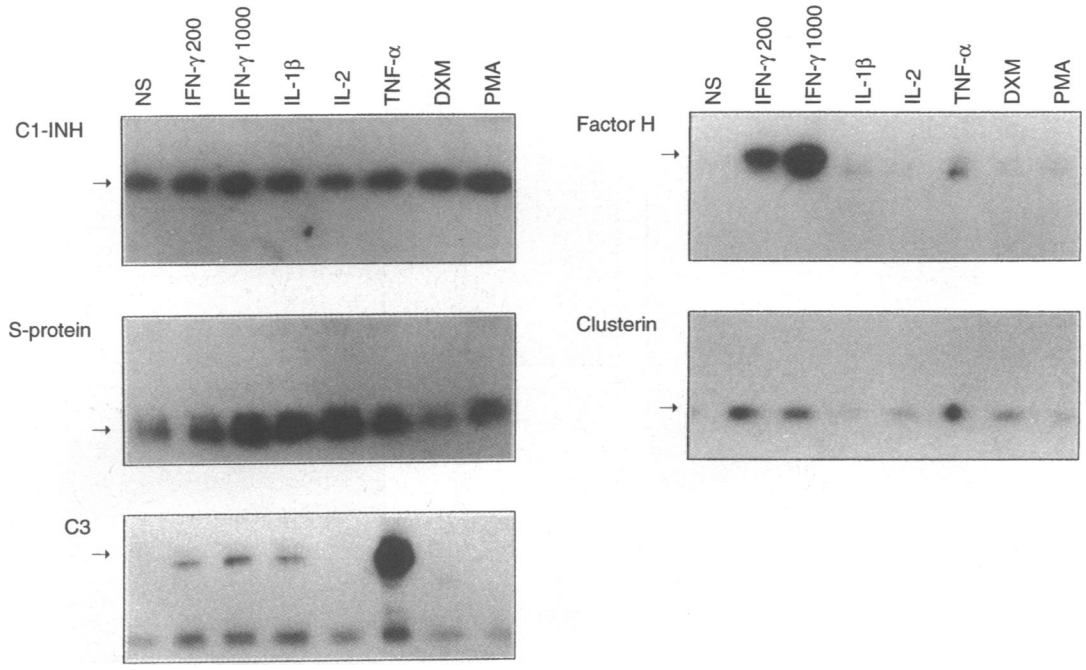

Figure 3. Expression of fluid-phase regulatory proteins by human oligodendrocyte cell line (HOG). Details of western blot analysis are given in the legend to Fig. 2. Cell culture supernatants of HOG unstimulated (NS) or after stimulation for 72 hr with IFN-γ (200 and 1000 IU/ml), IL-1β (100 IU/ml), IL-2 (100 IU/ml), TNF-α (1000 IU/ml), dexamethasone (DXM) (10^{-6} M) and PMA (10^{-6} M) were concentrated × 10 and analysed by western blot using: monoclonal anti-clusterin (G7) and rabbit antisera against S-protein, C1-INH, FH and C3. Human serum was used as a control to identify the correct molecular weight under non-reducing conditions (clusterin, 80 kDa; S-protein, 75 kDa; C1-INH, 110 kDa; FH, 140 kDa) and to permit quantitation by densitometry, allowing all results to be expressed as nanograms of protein per 10^6 cells (not shown).

cells and by western blotting of supernatants from cells cultured either with medium alone or with the stated cytokines. By immunostaining, permeabilized HOG were strongly positive for C1-INH, S-protein and weakly positive for clusterin (data not shown). By SDS–PAGE and western blotting of culture supernatants HOG were shown to constitutively secrete C1-inhibitor (110 kDa) (5 ± 3 ng/10^6 cells/48 hr), S-protein (75 kDa) (12 ± 7 ng/10^6 cells/48 hr), and at a very low level, clusterin (80 kDa) (< 1 ng/10^6 cells/48 hr) and factor H (140 kDa) (< 2 ng/10^6 cells/48 hr). C1-INH expression was not significantly affected after stimulation of HOG by various cytokines over a period of 72 hr, whereas the secretion of FH, clusterin and S-protein was enhanced by both IFN-γ and TNF-α (Fig. 3) and in a time- and dose-dependent manner (data not presented). IL-1 and IL-2 enhanced the expression of S-protein alone and dexamethasone had no effect on the expression of C inhibitors by HOG after 72 hr. C3 expression was used as an internal control for cytokine activities. We have previously reported that C3 is contitutively expressed by astrocytes and is upregulated by IL-1β and to a lesser extent by IFN-γ and TNF-α.[8] In contrast, HOG did not secrete C3 constitutively but C3 expression was inducible by IFN-γ, IL-1β and, most dramatically, by TNF-α.

Identification of mRNA for C regulators expressed by HOG

Expression of regulatory proteins by HOG, astrocyte cell line T98G and human fetal astrocyte was confirmed by RT-PCR (Fig. 4 and Table 1). No mRNA for CR1 was detected in HOG unstimulated or stimulated with PMA but a cDNA product at the correct size (514 bp) was identified in human fetal astrocyte and faintly in T98G (not presented) confirming the western blot result.

In contrast, all cells expressed abundant mRNA for MCP, DAF, CD59, S-protein and clusterin (Fig. 4, Table 1). C1-INH mRNA was easily detected in the HOG cell line whereas only a faint signal was observed for C4 bp in all cell lines and

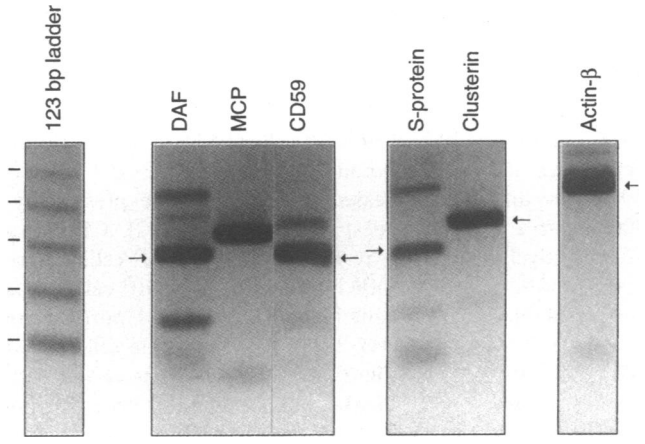

Figure 4. Analysis by RT–PCR of membrane and fluid-phase regulatory protein expression by human oligodendrocyte cell line (HOG). Analysis for DAF, MCP, CD59, S-protein and clusterin cDNA products obtained after RT–PCR of 1 μg of total RNA from unstimulated cells. Ethidium bromide staining of agarose gel (1·2%). A 123 bp ladder (Gibco) was used as a size marker: (from the bottom to the top: 123 bp, 246 bp, 369 bp, 492 bp, 615 bp).

unstimulated fetal astrocytes (Table 1). Actin-β RT–PCR was used as a positive control and to control for RNA loading throughout these studies.

DISCUSSION

We and others have previously shown that differentiated rat oligodendrocytes isolated from optic nerves spontaneously activate the classical pathway of C and lack the major MAC inhibitor CD59.[5,6,11,12] As a consequence of these two factors, rat oligodendrocytes are highly susceptible to killing by autologous serum. Rat oligodendrocyte adult progenitors and type II astrocytes are also able to activate C directly but are less sensitive since they can express the MAC inhibitor, CD59.[12] We are interested in ascertaining whether human oligodendrocytes are similarly susceptible to C but definitive studies are hampered by difficulties in obtaining highly pure cultures of these cells. Preliminary evidence indicates that cultured human oligodendrocytes do activate the classical pathway of C directly but, unlike rat oligodendrocytes, are not readily lysed by serum because they express CD59 on their surfaces.[23–25] In the present study we have used a well-characterized human oligodendrocyte cell line (HOG)[15–17] as a model to examine C activation by oligodendrocytes and the expression of membrane and fluid-phase C regulators by these cells.

HOG cells abundantly expressed CD59 and also expressed the membrane regulators MCP and DAF (though not CR1), demonstrating that the cells are well-protected from low-level 'bystander' C activation. Further, HOG cells secreted the fluid-phase C regulators C1-INH, S-protein and clusterin and fluid-phase forms of each of the expressed membrane inhibitors, providing an additional means of protection from 'bystander' activation. The molecular weight of each of the inhibitors was identical to that of the purified protein and/or that in control cells or serum (Fig. 2). CD59 and DAF on HOG cells were both GPI-anchored as evidenced by their efficient cleavage by PIPLC (Table 3). Expression of each of the regulators was confirmed at the mRNA level and again the RT–PCR products were of the predicted size and identical to controls (Fig. 4).

In both human and rat it has been reported that as yet uncharacterized myelin proteins directly fix C1q and induce activation of the C classical pathway.[13,14]

C activation on differentiated oligodendrocytes, both rat and human, is likely to involve a similar sequence of events in that C1q binds specifically to these cells to trigger C activation[24,26] but here too the receptor for C1q remains unknown. Human differentiated oligodendrocytes are, however, resistant to C killing[23–24] specifically because they express C inhibitors such as CD59. Neutralization of CD59 with a non-C-activating monoclonal antibody induced specific killing of human oligodendrocytes in the presence of NHS.[24] The HOG cell line displays characteristics typical of an immature undifferentiated oligodendrocyte in that it expresses several specific oligodendrocyte markers but does not constitutively express MBP or other myelin proteins.[16] In this study we show that HOG cells incubated in the presence of NHS failed to activate C as evidenced by maintenance of cell viability and the lack of binding of any of the C components (no binding of C1q, C4, C3 or MAC). Even neutralization of the major membrane inhibitors DAF and CD59 failed to expose any C activation, indicating that these cells, unlike rat and human oligodendrocytes, do not spontaneously activate C. We are currently investigating the capacity of HOG to express myelin proteins and to activate C after differentiation in culture and during co-culture with neuroblastoma or primary cultures of human fetal neurons.

Expression of DAF, MCP and CD59 on HOG were all enhanced by incubation with IFN-γ or PMA and the latter treatment also markedly increased the secretion of soluble forms of these proteins (Fig. 3). None of the cytokines or other stimuli tested induced the expression of CR1. IFN-γ also enhanced the secretion of S-protein and clusterin and induced the secretion of FH (Fig. 3). C3 secretion was used as a control in these studies and, although HOG cells did not constitutively secrete C3, they were induced to do so by incubation with IFN-γ, IL-1β and, most markedly, by TNF-α (Fig. 3). Complement component biosynthesis by HOG cells is thus worthy of further investigation.

Among the control cells used in this study were primary human astrocytes and astrocyte-derived cell lines. We have previously reported expression of C regulatory molecules by these cells at the mRNA level.[10] We here confirm these findings and extend them to the protein level. Astrocytes and astroglioma lines expressed the membrane inhibitors MCP, DAF and CD59 at levels equivalent to or higher than those on HOG cells and also expressed CR1 which is not present on HOG (Table 2 Fig. 2).

Soluble forms of the membrane inhibitors MCP, DAF and CD59 were detected in cell culture supernatants from HOG (Fig. 2) astrocytes and astrocyte cell lines (not shown), particularly after PMA stimulation. The presence of soluble forms of these C inhibitors has been reported in plasma[27–29] and also within the cerebrospinal fluid of healthy individuals.[25] The likely source of these proteins *in vivo* is the normal turnover of membrane proteins from epithelia and circulating cells and glia may represent the major source for cerebrospinal fluid. The functional relevance of these fluid-phase forms of the membrane inhibitors in plasma or cerebrospinal fluid, either as C inhibitors or in other contexts, remains to be demonstrated.

If HOG cells and cultures of human oligodendrocytes *in vitro*[23–25] are appropriate models for human differentiated and myelinating oligodendrocytes *in vivo* then a prediction of the present study would be that oligodendrocytes *in vivo* are C activating but are well-protected from 'bystander' C attack. Pro-inflammatory cytokines, notably IFN-γ, enhanced expression of the majority of the membrane and fluid-phase inhibitors by HOG. Thus, at inflammatory sites oligodendrocytes would be predicted to be even more resistant to C-mediated damage. Immunohistological evidence indicates that oligodendrocytes *in situ* in normal brain express C inhibitors including CD59 at very low levels or not at all[24,30–33] and it has been suggested that this is a consequence of a downregulation of C inibitor expression on all glial cells in the normal CNS which may be reversed in inflammation.[24,25]

Expression of other C inhibitors has previously been little studied in brain. Despite the *in vitro* findings reported here and elsewhere[23–25] it remains possible that unstimulated oligodendrocytes *in vivo* are relatively inefficient at regulating C activation. In the normal brain the blood-brain barrier (BBB) is intact and there is no transudation of plasma (source of C) into the brain tissue. It has been reported, however, that the early components of C (C1q, C3 and C4) but not the late components (C7 and C9) are expressed *in vivo* at the mRNA level in normal

brain, probably by microglia, astrocytes and perhaps even neurons.[34–36] A partial C system can thus be produced locally by cells in normal brain but probably in a restricted manner, at a low level and without the possibility to form a cytotoxic MAC, and this may be correlated with the low expression of C inhibitors by putative target cells such as oligodendrocytes and neurons *in vivo*.[32–33] During an autoimmune disease such as multiple sclerosis C will be present in the brain either through breakdown of the BBB or through the induction or upregulation of intrathecal synthesis by brain cells. In particular, release of cytokines such as IFN-γ, IL-1β and TNF-α by infiltrating immune cells will have pronounced effects to upregulate or induce C biosynthesis by brain cells.[8–10] Myelin will be exposed on damaged mature oligodendrocytes and this will initiate C activation with consequent cytotoxicity and cytolysis of oligodendrocytes.[37] Enhanced expression of C inhibitors by oligodendrocytes, triggered by inflammatory cytokines, notably IFN-γ, will contribute to C inhibition and protection of those cells not killed in the initial assault. Inhibitors of C may thus be of value in preventing loss of oligodendrocytes, as has already been demonstrated in animal models of demyelination.[38]

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