Spontaneous Classical Pathway Activation and Deficiency of Membrane Regulators Render Human Neurons Susceptible to Complement Lysis

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This study investigated the capacity of neurons and astrocytes to spontaneously activate the complement system and control activation by expressing complement regulators. Human fetal neurons spontaneously activated complement through the classical pathway in normal and immunoglobulin-deficient serum and C1q binding was noted on neurons but not on astrocytes. A strong staining for C4, C3b, iC3b neoepitope and C9 neoepitope was also found on neurons. More than 40% of human fetal neurons were lysed when exposed to normal human serum in the presence of a CD59-blocking antibody, whereas astrocytes were unaffected. Significant reduction in neuronal cell lysis was observed after the addition of soluble complement receptor 1 at 10 µg/ml. Fetal neurons were stained for CD59 and CD46 and were negative for CD55 and CD35. In contrast, fetal astrocytes were strongly stained for CD59, CD46, CD55, and were negative for CD35. This study demonstrates that human fetal neurons activate spontaneously the classical pathway of complement in an antibody-independent manner to assemble the cytolytic membrane attack complex on their membranes, whereas astrocytes are unaffected. One reason for the susceptibility of neurons to complement-mediated damage *in vivo* may reside in their poor capacity to control complement activation. (Am J Pathol 2000, 157:905–918)

Complement is part of the body's natural immune defense system and comprises >30 secreted and cell-bound proteins. Some of these proteins belong to the complement pathways whereas others function as regulators. Complement activation occurs in a sequential manner via the classical pathway involving C1 (C1q, C1r, C1s), C4, C2, and C3, or the alternative pathway involving C3, factor B, factor D, and properdin.^{1,2} One of the most remarkable properties of complement is its capacity

to lyse targeted organisms, such as bacteria, viruses, and parasites.^{3–5} This involves the assembly of the late complement components C5, C6, C7, C8, and C9 to form the lytic membrane attack complex (MAC). After inappropriate activation, for example on self-cells, complement regulatory proteins act at various stages to block the cascade of activation. These regulators can be fluid-phase proteins such as C1 inhibitor, C4b binding protein, factor H, factor I, S-protein, and clusterin, or membrane-bound proteins such as membrane cofactor protein (MCP, CD46), decay accelerating factor (DAF, CD55), complement receptor 1 (CR1, CD35), and CD59.^{6–8}

Transudation of complement proteins through a damaged blood-brain barrier can contribute to the deposition of potentially cytolytic components of the complement pathway on the surface of neurons and glial cells. In support of this, a number of reports exist implicating complement-mediated damage in the etiology of neurodegenerative disease, demyelinating disease, and ischemic stroke.^{9–18} Mechanisms involved in the activation of complement in neurodegeneration are complex, but in Alzheimer's disease there is evidence for the activation of the classical pathway of complement by β /A42 fibrils present within the amyloid plaques.^{19,20} Additionally, local biosynthesis of complement components by activated glial cells such as astrocytes and microglia has been reported and may also contribute to neuronal loss if complement activation is uncontrolled.^{21–24}

One important factor influencing the vulnerability of cells to spontaneous complement activation and cell lysis is the expression of membrane-bound complement regulators such as CR1, DAF, MCP, and CD59. Others have addressed the expression of complement regulatory proteins on primary cultures of human brain cells *in vitro* but have concentrated on astrocytes^{25,26} and oligodendrocytes.^{27,28} It is reported that astrocytes express MCP, CD59, and low levels of DAF whereas human oligodendrocytes express only DAF.²⁸ The capacity of rat oligodendrocytes to directly activate the complement system

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in vitro has long been reported^{29–31} and their vulnerability to complement-mediated lysis was attributed to the lack of CD59 on their membranes.³² Rat type II astrocytes were also noted to spontaneously activate the complement system but were shown to express CD59 and hence were resistant to complement-mediated lysis *in vitro*.³²

Complement activation and expression of complement regulatory proteins on neurons has received little attention. Human neuroblastoma cell lines (IMR32, SKN-SH, differentiated NT2, and SH-SY5Y) have been reported to spontaneously activate the classical pathway of complement.^{33–35} The majority of these cell lines expressed low levels of CD59, lacked DAF, and were consequently lysed when cultured in the presence of human serum. The SH-SY5Y line expressed CD59 and was lysed by serum only after enzymatic removal of CD59.³⁵ Primary cultures of human neurons have not been investigated for their ability to spontaneously activate the complement system and to control complement activation by expressing complement regulators. We have performed a systematic investigation to test whether human fetal neurons spontaneously activate the complement system. We also assessed their capacity to inhibit the complement cascade at the C3/C5 convertase stage and at the stage of MAC formation by expressing membrane-bound complement regulators. We extended our study to characterize the expression of complement regulatory proteins at the mRNA level using both reverse transcriptase-polymerase chain reaction (RT-PCR) and *in situ* hybridization (ISH) on human fetal brain cells.

Materials and Methods

Primary Mixed Human Fetal Brain Cell Culture

Fresh human fetal brain tissue from 10 samples (10- to 12-week-old fetus) was received in Hanks' buffer from the Medical Research Council Tissue Bank (Dr. Wong, Hammersmith Hospital, London, UK) and used for cell cultures following local ethical guidance (BRO TAF Health Authority, Ref 98/2773). The brain tissue was chopped into 1-cm³ pieces, washed twice in fresh Hanks' buffer (Sigma, Poole, Dorset, UK), and pelleted by centrifugation at 1,000 rpm. The tissue was digested in 0.05% trypsin (Sigma) in Hanks' buffer at 37°C for 15 minutes with constant agitation and the mixture was treated with DNase I (0.2 µg/ml final; Sigma) for 1 minute. Cells were thoroughly dissociated using a 3-ml plastic Pasteur pipette and finally resuspended in complete medium that consisted of Dulbecco's modified Eagle's medium (Gibco Life Technologies, Paisley, UK) supplemented with heat inactivated 10% fetal calf serum, 4 mmol/L glutamine, 2.5 µg/ml fungizone, 2 mmol/L sodium pyruvate, 100 IU/ml penicillin, and 100 IU/ml streptomycin. The cells were filtered through a 70-µm nylon cell strainer (Becton-Dickinson, Cowley, Oxford, UK) and pelleted by centrifugation at 1,500 rpm for 20 minutes. The cell pellet was resuspended in fresh complete medium further supplemented with 2.5 mmol/L KCl to encourage neuronal cell growth.³⁶ Cells were seeded at 10⁷ cells/10 ml in

25-cm² culture flasks coated with 10 µg/ml poly-L-lysine (Sigma). Primary mixed cell cultures were also seeded on coverslips. Coverslips were first coated with a 2% solution of 3-aminopropyltriethoxysilane (Sigma) in acetone for 5 minutes followed by poly-L-lysine as for flasks. Coverslips were individually placed into 12-well plates. Cells in complete medium (200 µl, 10⁶ cells/ml) were transferred onto each coverslip and incubated in 95% air/5% CO₂ in a humidified incubator at 37°C.

Source of Antibodies

The sources of polyclonal antibodies were: rabbit anti-C1q (OTNT05) from Behring Diagnostics (Hamburg, Germany); rabbit anti-C4 from Sigma; rabbit anti-C3c obtained as a gift from Dr. M. Fontaine (INSERM U519, Rouen, France); and rabbit anti-factor B from Serotec (Oxford, UK). The mouse monoclonal antibodies (mAb) against complement were: anti-iC3b neoeptide from Quidel (San Diego, CA); clone C3/30 anti-C3b, a gift from Dr. P. W. Taylor (Ciba-Geigy Ltd., Horsham, UK); and mAb clone B7, anti-C9 neoeptide (raised in-house). The mouse anti-C1q (clone 12A5B7) hybridoma was purchased from the American Type Culture Collection (Rockville, MD). Rabbit anti-glial fibrillary acidic protein (GFAP, code B5) and mouse mAb anti-GFAP (clone MCAB5.2E4) were from Dr. J. Newcombe (Multiple Sclerosis Society Laboratory, London, UK). Rabbit antisera against CR1, DAF, and MCP were all raised in-house using highly purified or recombinant proteins as immunogens. The specificity of all antibodies against complement components and complement regulators was further tested by Western blot analysis using either human serum or cell lines such as HeLa, THP1, K562, CB193, and IMR32.³⁷ Mouse mAb anti-MCP (clone GB24) was from Professor J. P. Atkinson (Washington University School of Medicine, St. Louis, MO). Mouse mAb OX23 anti-human complement factor H was from Dr. R. B. Sim (Medical Research Council Unit, Oxford, UK). Mouse mAb anti-neuron-specific enolase (NSE) clone BBS/NC/VI-H14 was from DAKO Ltd., (Milton Keynes, UK). Mouse mAb anti-NCAM (CD56) clone MY31 was from Becton-Dickinson. Mouse mAbs anti-CD44 (clone BRIC 222), anti-DAF (CD55) (BRIC 216), and anti-CD59 (BRIC 229) were all purchased from the International Blood Group Reference Laboratory (Oxford, UK). Rabbit anti-kappa (κ) and rabbit anti-lambda (λ) antisera were purchased from ICN Pharmaceuticals Ltd. (Oxford, UK).

Identification of Cells in Primary Human Fetal Brain Culture

Measurement of Complement-Mediated Lysis of Neurons in Human Fetal Brain Primary Cell Cultures using Calcein and Propidium Iodide (PI)

Mixed human fetal brain cells were cultured on glass coverslips in a 12-well plate. Neurons were easily identified as small rounded cells co-culturing in the presence of astrocytes (large flat cells) and their identity was con-

firmed by immunocytochemistry using specific cell markers (anti-NSE and anti-GFAP) as described previously.^{38,39} Cells were loaded for 1 hour at 37°C with the green fluorescent dye calcein.AM (Molecular Probes, Eugene, OR) diluted in complete medium to 2 $\mu\text{g/ml}$ final concentration. Inside the cell, calcein.AM is de-esterified to a polar fluorescent product which is retained within intact cells and released only after membrane damage (cell killing). Cells were washed three times in 0.9% sterile saline (tissue culture grade) and incubated in veronal buffer (VBS; Oxoid Ltd., Basingstoke, UK) containing 1% bovine serum albumin (BSA) and PI at 10 $\mu\text{g/ml}$ final concentration for 30 minutes or 1 hour at 37°C containing the following: 1) normal human serum (NHS) diluted (1/4 or 1/8); 2) heat-inactivated NHS (inactivation at 56°C for 30 minutes) at the same dilutions; 3) NHS diluted 1/8 in VBS/BSA to which was added the noncomplement-fixing but neutralizing antibody against CD59 (mouse IgG2b isotype, clone BRIC229) at 13 $\mu\text{g/ml}$; 4) VBS/BSA containing mouse anti-CD59 antibody (clone BRIC229) at 13 $\mu\text{g/ml}$; and 5) NHS diluted 1/8 in VBS/BSA containing mouse anti-CD59 antibody (clone BRIC229) at 13 $\mu\text{g/ml}$ and soluble complement receptor 1 (sCR1; T cell Sciences, Needham, UK) at 10 $\mu\text{g/ml}$ final concentration.

The coverslips were inverted onto prelabeled glass microscope slides and the cells examined under a fluorescent microscope (Leica UK Ltd., Milton Keynes, UK) using the fluorescein isothiocyanate filter for the calcein signal and the rhodamine filter for the PI signal. Lysed cells were PI-positive (depicted as white spherical cells) and calcein-negative (because of leakage of the green fluorochrome). Viable cells were green fluorescent (visualized as gray cells) because of calcein retention and PI-negative and are illustrated in Figure 2A.

Immunocytochemistry for Detection of Complement Activation and Complement Regulators on Human Fetal Brain Primary Cell Cultures

Mixed human fetal brain cells cultured on coverslips were incubated for 30 minutes at 37°C with NHS diluted 1/16 in VBS/1%BSA or with NHS deficient in IgA, IgM, and IgG (Sigma) diluted 1/8. Controls included heat-inactivated NHS and NHS containing 10 mmol/L ethylenediaminetetraacetic acid (EDTA). The cells were thoroughly washed five times in phosphate-buffered saline (PBS), pH 7.3, and fixed in acetone for 5 minutes at room temperature followed by further washes (5 times) in PBS. All coverslips were immersed for 30 minutes in PBS/BSA to block the nonspecific-antibody binding. The coverslips were incubated in 100 μl of the appropriate dilution of primary antibody (anti- κ and anti- λ light chains, anti-complement, or anti-complement regulatory protein) at 4°C overnight in a humidity chamber. The cells on coverslips were thoroughly washed (10 times) in PBS and incubated for 1 hour at room temperature in the specific secondary peroxidase conjugate (Bio-Rad, Hemel Hempstead, UK) diluted 1/100 in PBS/BSA. Cells were washed 10 times in

PBS after which they were developed for 5 minutes in a freshly-made solution of 0.05% diaminobenzidine (DAB) and 0.005% (v/v) hydrogen peroxide diluted in PBS. After a brief wash in PBS, the cells were washed thoroughly in water before and after counterstaining in hematoxylin. After either a full dehydration in ethanol or air-drying (37°C oven), the cells were cleared in xylene and the coverslips were mounted on glass slides.

The level of immunostaining on fetal brain cells using anti-complement antibodies was assessed by semiquantitative image analysis (Openlab/Improvision, Coventry, UK). The salient instrumentation of the system included a color digital camera mounted on a light microscope and connected to a computer based image analysis system. Briefly, the method involved recording a series of images of the areas of interest (neurons and astrocytes) in the sample and measuring the amount of DAB (brown) staining of gated cells from 10 random but representative fields in each sample. The data were sorted and expressed as a mean $\pm$ SEM of the measurements (staining index).

For double-immunocytochemistry, antibodies to GFAP and anti-complement regulatory protein derived from different species were simultaneously applied to cells on coverslips. The secondary conjugates specific for each of the primary antibodies were peroxidase-conjugated goat anti-mouse/rabbit immunoglobulins diluted 1/100 (Bio-Rad) and alkaline phosphatase conjugated goat anti-mouse/rabbit immunoglobulins diluted 1/500 (Sigma). The substrates were: 0.05% DAB/0.005% hydrogen peroxide diluted in PBS and nitro blue tetrazolium (NBT)/5-bromo-4-chloro-3-indolyl phosphate (BCIP). NBT stock solution was at 75 mg/ml dissolved in 70% *N-N*-dimethyl formamide and BCIP stock solution was at 50 mg/ml dissolved in *N-N*-dimethyl formamide. A fresh solution of alkaline phosphatase substrate was prepared by adding 4.5 μl of stock NBT and 3.5 μl BCIP per 1 ml of detection buffer (0.1 mol/L Tris-HCl, 0.1 mol/L NaCl, 50 mmol/L MgCl_2 , pH 9.5) to which 0.3% levamisole (Sigma) was added. The co-localization of the brown (DAB) and blue (NBT/BCIP) product was identified on the cells. Representative bright-field images were photographed using a Leica DMLB microscope (Leica UK Ltd.).

Flow Cytometric Analysis of Human Fetal Brain Primary Cell Cultures

The detection of membrane-bound complement regulators (CR1, DAF, MCP, CD59) and cell markers (CD44, CD56) on mixed human fetal brain cells was assessed by flow cytometry. All steps were performed on ice. Cultured adherent fetal brain cells were washed three times in sterile NaCl (0.9%) and harvested using 10 mmol/L EDTA in PBS/BSA. The cells (10^5 cells/tube) were incubated in primary antibody at the appropriate dilution in PBS/BSA for 1 hour, washed three times in PBS/BSA by centrifugation (1,000 rpm for 3 minutes) before incubation for 1 hour with the secondary red phycoerythrin-conjugated antibody (DAKO, High Wycombe, Bucks, UK) diluted in PBS/BSA. The cells were washed three times in PBS/BSA

A

Figure 1. Complement activation and opsonization on human fetal neurons cultured in the presence of NHS. **A:** A 5-day primary mixed-cell culture (astrocytes and neurons) from human fetal brain was incubated (30 minutes at 37°C) with either VBS/BSA (**a, c, e, g, i**) or NHS diluted 1/16 (**b, d, f, h, j**). Complement activation and opsonization were detected by the immunoperoxidase/DAB method. Cells were lightly counterstained in hematoxylin to show nuclei (blue) of cells. Original magnification, $\times 550$. **a** and **b:** Mouse anti-C1q. Control cells (incubation in VBS/BSA) were not stained for C1q whereas neurons (**large arrowhead**) incubated with NHS were stained for C1q. Astrocytes (**long arrow**) remained immunonegative for C1q. **c** and **d:** Rabbit anti-C4. The staining was intense on neurons (**short arrows**) and weak on astrocytes (**long arrows**) after treatment with NHS (**d**). Control cells were C4-negative. **e** and **f:** Mouse anti-iC3b neopeptide. Strong membranous staining was detected on neurons incubated with NHS (**short arrow**) and occasionally very weak staining was observed on astrocytes (**long arrow**). Control cells (VBS/BSA) were negative for iC3b. **g** and **h:** Mouse anti-C3b. The staining was strong on neurons (**short arrow**) but absent on astrocytes (**long arrow**) after incubation with NHS (**h**). Control cells were negative. **i** and **j:** Mouse anti-C9 neopeptide (MAC). Neurons (**short arrow**) but not astrocytes (**long arrow**) were stained for MAC. Control cells were MAC-negative. **B:** Semiquantification of the intensity of staining using the image analysis station from Openlab (see text). The staining index was measured on gated neurons and astrocytes after immunostaining with anti-complement antibodies—rabbit anti-C1q (C1q), rabbit anti-C4 (C4), rabbit anti-C3 (C3), mouse anti-iC3b (iC3b), and mouse anti-C9 neopeptide (C9neo). Results are mean of staining $\pm$ SEM ($n = 10$).

as before and analyzed on a flow cytometer (Becton-Dickinson). Two distinct populations present in the mixed human fetal brain were identified from the cell scatter (FSC/SSC plot). The small cells (38 to 40% of the total cell population) were CD56^{high}, CD44^{dim}, and GFAP^{negative} and were identified as human fetal neurons. The large cells were CD56^{high}, CD44^{high}, and GFAP^{high} and represented the fetal astrocyte population as already described.^{40,41}

RT-PCR to Detect the mRNA for Complement Regulators

Total RNA was isolated from four different samples of human fetal brain primary cell cultures using the Ultraspec RNA isolating reagent according to the manufacturer's instructions (Biotecx Labs., Houston, TX). Reverse transcription (RT) was performed at 37°C for 2 hours using 3 μ g of total RNA in the presence of 50 mmol/L Tris-HCl, 75 mmol/L KCl, 3 mmol/L MgCl₂, 5 μ mol/L dithiothreitol, 60 U rRNasin, and 2 mmol/L dNTPs and MMLV in a total volume of 30 μ l. A 3- μ l aliquot of RT reaction was used for polymerase chain reaction (PCR) using specific oligonucleotide pairs (see below) for each

of the complement regulators. Their gene target, primer sequence, and the predicted size of each cDNA product are: MCP (GCTACCTGTCTCAGATGACG) (ACCACTT-TACTACTCTGGAGC) 419 bp; CR1 (TGGCATGGTGCATGTGATCA) (TCAGGGCCTGGCACTTCACA) 514 bp; DAF (GCAACACGGAGTACACCTGT) (GCTAAGAATGTGATTCCAGG) 360 bp; clusterin (GTCTCAGACAATGAGCTCCA) (TGCGGTCCACCATTATCCAG) 419 bp; CD59 (ATTTCAACGACGTCACAACC) (GACTGGTCTCAAAGTCTCC) 369 bp; glyceraldehyde phosphate dehydrogenase (GAPDH) (GAACGGGAAGCTTGTCATCA) (TGACCTTGCCACAGCCTTG) 473 bp.

PCR amplifications were performed in an Omnigene thermocycler (Hybaid, Teddington, UK) using the following conditions: denaturation at 94°C for 4 minutes, five cycles (94°C for 30 seconds, annealing 60°C for 1 minute, 72°C extension for 2 minutes), 20 cycles (94°C for 30 seconds, annealing 60°C for 30 seconds, 72°C extension for 45 seconds), and a final extension at 72°C for 15 minutes. All samples were subjected to RT-PCR for housekeeping gene GAPDH as a positive control and as an internal standard.

RT-PCR products were resolved on 1.2% agarose gels in 1 $\times$ Tris-borate-EDTA (TBE) buffer. Comparative DNA ladder markers (123 bp and 1 kb from Life Technologies Ltd.) were loaded to identify the correct size of the different cDNA fragments. Gels were visualized by ethidium bromide and photographed (using a gel 1,000 UV documentation system, Bio-Rad). The identity of each of the cDNA fragments was confirmed by sequencing using the Big DYE sequencing kit (Perkin Elmer, Buckinghamshire, UK) and analysis on the ABI 377 automated sequencer (Perkin Elmer).

ISH to Identify the Differential Expression of Complement Regulators by Human Fetal Brain Cells

Plasmid containing the full-length human MCP cDNA clone was used with appropriate primer pairs (see above) to generate a specific riboprobe of 419 bp for use in ISH on mixed fetal brain cultures. The PCR product corresponded to bases +301 to +720 in the MCP coding region^{39,42} (accession number Y00651) and was cloned into pGEM-T (Promega, Southampton, UK) with flanking SP6/T7 RNA polymerase sites. The human CD59 riboprobe was a 518-bp PCR product containing the region -37 to +481 (accession number M95708) cloned into pGEM-3Z (Promega).⁴³ A 473-bp PCR fragment of GAPDH cDNA coding region +249 to +702 (accession number M33197) was also cloned into pGEM-T. In all cases, the identity and orientation of the cloned fragments were confirmed by sequencing with T7 and SP6 primers.

The digoxigenin-UTP labeling kit was purchased from Boehringer Mannheim (SP6/T7; Lewes, East Sussex, UK). Both sense and antisense riboprobes were generated by *in vitro* transcription of the linearized plasmid (1 μ g) using either SP6 or T7 RNA polymerase. The level of digoxige-

A

B

nin incorporation was assessed according to the manufacturer's instructions.

Primary mixed human fetal brain cultures growing on glass coverslips were washed gently in PBS and permeabilized with 5 μ g/ml proteinase K in Tris/EDTA buffer (100 mmol/L Tris/50 mmol/L EDTA, pH 8.0) for 40 minutes at 37°C. Enzymatic digestion was terminated by immersing the coverslips in freshly prepared 4% paraformaldehyde, pH 7.5, for 10 minutes. Two further washes (10 minutes each) were performed in DEPC-treated PBS. The cells were allowed to dry at 37°C for 1 hour.

Coverslips were coated in the hybridization solution (100 μ l per coverslip) which consisted of the digoxigenin-UTP labeled riboprobe (20 ng/ μ l) in 50% formamide, 4 $\times$ standard saline citrate, 1 $\times$ Denhardt's solution, 10% dextran sulfate, 0.05 mg/ml denatured salmon sperm DNA, 1% *N*-lauroylsarcosine, 0.02 mol/L Na₂PO₄ (pH 7.0), and 50 mmol/L dithiothreitol. The incubation was performed overnight at 50°C. Coverslips were washed (10 $\times$ 1 minute) at 37°C using prewarmed 1 $\times$ standard saline citrate. Further washes 2 $\times$ 10 minutes in buffer C (100 mmol/L Tris, pH 7.5, 150 mmol/L NaCl, 0.2% Tween 20) were performed before incubation overnight at 4°C in buffer C containing 1.5% BSA.

The coverslips were incubated at 4°C with sheep anti-digoxigenin Fab fragments conjugated to alkaline phosphatase (Boehringer Mannheim) diluted 1/5,000 in antibody diluting buffer (100 mmol/L Tris, pH 7.5, 850 mmol/L NaCl, 0.2% Tween 20). The coverslips were washed 10 $\times$ 1 minute each in fresh buffer C followed by a brief wash in detection buffer. The alkaline phosphatase substrate diluted in detection buffer (NBT/BCIP; see immunocytochemistry above) containing 0.3% of levamisole was applied to coverslips at room temperature. The color development was allowed to take place in the dark for 6 to 10 hours. The reaction was monitored periodically by light microscopy. Development was stopped by thoroughly washing cells in distilled water. The cells were allowed to dry at 37°C for 1 hour, then cleared in xylene, and

Figure 2. Complement-mediated lysis of human fetal neurons using primary culture of mixed cells (astrocytes and neurons). Human mixed cultures of fetal brain were loaded with the green fluorescent dye calcein. **A:** Cells were incubated for 30 minutes with: VBS/BSA containing anti-CD59 antibody at 13 μ g/ml (**a**); NHS diluted 1/8 in VBS/BSA and containing mouse anti-CD59 (**b**); NHS diluted 1/8 containing anti-CD59 but with sCR1 added at 10 μ g/ml (**c**). The red fluorescent dye, PI, was added to stain the nuclei of lysed cells. Cells were examined using the fluorescein isothiocyanate filter for calcein fluorescence and the rhodamine filter for PI. Live cells remained calcein-positive (gray cells in all fields) and nuclei were not stained. In contrast, dead cells did not retain calcein and the nuclei were PI-positive. In **a**, the majority of the neurons (**small arrows**) and astrocytes (**large arrow**) were calcein-positive. In **b**, there is near-complete lysis of neurons with only the occasional neuron being spared (**small arrow**). Astrocytes remained unaffected (**large arrow**). In **c**, lysis of human fetal neurons was reduced compared with **b**. All images in black and white are at original magnification, $\times 250$. **B:** Eight random frames for each treatment described were photographed for semi-quantitative estimation of complement-mediated lysis. The presence of PI-positive cells (white-stained nuclei) was assessed by fluorescent microscopy. The average number ($\pm$ SD) of lysed cells were estimated by automated counting of white nuclei and the data expressed in graphic form. The control containing VBS/BSA and anti-CD59 shows minimal lysis after 30 minutes of incubation. However, neuronal lysis was apparent after subjecting the mixed CNS cells to NHS (1/8, for 30 minutes) containing the anti-CD59 neutralizing antibody. The cells treated for 30 minutes with NHS 1/8 plus CD59 antibody and sCR1 (10 μ g/ml) showed a significant reduction in the numbers of lysed cells.

Figure 3. Immunodetection of complement regulators on human fetal brain mixed cell culture. Five-day-old primary mixed cell culture from human fetal brain were double-immunostained for complement regulators (CR1, DAF, MCP, CD59) and specific cell markers (CD56, GFAP) using the immunoperoxidase/DAB and immunoalkaline phosphatase/NBT/BCIP protocols. Original magnification, $\times 260$. **a:** Staining using a rabbit anti-GFAP combined with a mouse irrelevant antibody to human factor H, clone OX23. Astrocytes (**long arrow**) but not neurons (**short arrow**) were stained for GFAP (brown staining). No blue staining was detected with the irrelevant mouse antibody. **b:** Rabbit anti-GFAP/mouse anti-CD56. Astrocytes (**long arrow**) were stained for both markers, whereas neurons (**short arrows**) were stained only for CD56. **c:** Mouse anti-GFAP/rabbit anti-CR1. CR1 (blue) staining was absent on astrocytes (**long arrow**) and neurons (**short arrow**). **d:** Mouse anti-GFAP/rabbit anti-DAF. Blue immunostaining of DAF is clearly detected on the astrocytes (**long arrow**). The dark brown appearance is because of the co-localization of the blue DAF staining with the light brown GFAP staining. The neurons (**short arrow**) are clearly negative for both GFAP and DAF. **e:** Mouse anti-GFAP/rabbit anti-MCP. Astrocytes were stained for GFAP and MCP whereas neurons were weakly stained only for MCP. **f:** Rabbit anti-GFAP/mouse anti-CD59. CD59 staining (blue) was detected on both cell types.

Figure 4. Fluorescence-activated cell sorting analysis of mixed human fetal brain cells stained for membrane complement regulators. Human fetal brain cells were stained with various antibodies to complement regulators and red phycoerythrin-conjugated secondary antibody (measured in FL2 channel). The distribution and the intensity of the staining (histogram depicting intensity of fluorescence *versus* cell count) were obtained by gating the neuron and the astrocyte populations identified from the cell scatter plot. Results from the gated populations of neurons (**a**) and astrocytes (**b**) are presented. The control histogram (gray) was obtained after staining with an irrelevant antibody (rabbit anti-factor H). The black histogram was obtained after staining with mouse anti-CD44 (**1a, b**); rabbit anti-CR1 (**2a, b**); mouse anti-DAF clone BRIC 216 (**3a, b**); mouse anti-MCP clone GB24, (**4a, b**) and mouse anti-CD59 clone BRIC 229 (**5a, b**). No specific staining was obtained with the mouse anti-factor H irrelevant antibody (clone OX23; data not shown).

Figure 5. Expression of complement regulator mRNAs by human fetal brain cells: RT-PCR analysis. Total RNA was prepared from 5-day-old primary mixed culture of human fetal brain. RT-PCR was performed for MCP, CR1, DAF, CD59, clusterin, and GAPDH mRNAs. The cDNA products were resolved on an ethidium bromide stained 1.2% agarose gel and examined and photographed using the Bio-Rad Gel Doc1000 station. The identity of each cDNA was confirmed by sequencing (see text). The size in bp of the DNA ladder (1 kb, Gibco BRL) is indicated on the left-hand margin.

mounted in a neutral mounting medium before detailed examination under the light microscope.

Results

Human Fetal Neurons Spontaneously Activate the Classical Pathway of Complement

Primary fetal brain cultures contained predominantly two types of cells. The numerous small cells were neurons immunopositive with anti-NSE. These cells were closely associated with large, flat cells which unequivocally stained for GFAP indicating that they were astrocytes.^{38,39} Complement components and activation products of the classical pathway (C1q, C4, C3b, iC3b, C3c) were detected specifically on fetal brain cells after incubation for 30 minutes at 37°C in NHS. The distribution of the complement immunostaining on neurons and astrocytes is illustrated in Figure 1A; the level of staining was quantified on gated cells and is presented in Figure 1B. Anti-C1q staining was detected on neurons (Figure 1A; b, short arrow) but not on astrocytes (Figure 1A; b, long arrow). Anti-C4 stained neurons intensely (Figure 1A; d, short arrows) with either little or no staining of astrocytes (Figure 1A; b, long arrows). Monoclonal antibodies to C3 activation products, iC3b (Figure 1A; f, short arrow) and C3b (Figure 1A; h, short arrow) bound strongly to neuronal membranes. The presence of the MAC on neurons (Figure 1A; j, short arrow) was detected with a C9 neoepitope-specific monoclonal antibody (clone B7). Astrocytes were free of immunostaining for MAC using the same antibody (Figure 1A; j, long arrow). After complement activation, image analysis using the Openlab/Improvision software allowed the semiquantitation of the intensity of immunostaining both on neurons and astrocytes using the same samples as those shown in Figure 1A. The level of staining with each antibody was high on neurons and weak or absent on astrocytes (Figure 1B).

Immunostaining with anti-factor B was negative throughout (data not shown). No staining for complement components was detected on control cells treated with VBS/BSA (Figure 1A: a, c, e, g, and i) or with NHS containing 10 mmol/L EDTA (data not shown). Rabbit anti-human κ light-chain immunoglobulins weakly stained human fetal neurons whereas astrocytes were negative (data not shown). To test whether neurons activate spontaneously the complement system in an antibody-independent manner, cultured cells were incubated with NHS deficient in IgA, IgM, and IgG and then stained for complement opsonization. No binding of immunoglobulins (κ and λ) was detected on neurons and astrocytes (data not shown). However, a strong staining for C1q, C4, C3, and C9 was detected on neurons but not on astrocytes (data not shown).

Human Fetal Neurons but Not Astrocytes Are Lysed after Spontaneous Complement Activation

Mixed human fetal brain cells loaded with calcein were incubated for 30 minutes at 37°C with VBS/BSA/PI alone or with NHS (Figure 2A). The level of complement-mediated lysis was examined by identifying and counting the number of calcein-negative cells represented as white spherical cells from eight randomly selected microscope fields of view (Figure 2A). Lysed calcein-negative neurons were infrequent (Figure 2A; a) in control cultures (treated with heat-inactivated serum diluted 1/8 or with VBS/BSA) and astrocytes were not lysed (Figure 2A; a, long arrow). Few neurons were lysed (white spherical cells) in the first 30 minutes after treatment with NHS diluted 1/8 whereas almost all neurons were lysed after 1 hour to 2 hours after incubation with NHS diluted 1/8. Moreover, almost all neurons were lysed after complement activation using NHS (diluted 1/8, 30 minutes after incubation) to which a noncomplement-fixing, but neutralizing, mouse anti-CD59 (BRIC229) was added (Figure 2A; b). Astrocytes (large gray cells) remained viable after the same treatment. Lysis of neurons (number of white spherical cells) was significantly reduced (Figure 2A, c; and B) on the addition of sCR1 (10 μ g/ml) to the cultures incubated with NHS (1/8) containing the anti-CD59 antibody.

Semiquantitative estimation of complement-mediated lysis of human fetal neurons was also performed. The numbers of PI-positive cells (white spherical cells) were counted from eight random microscope fields of view (on photographs) and the mean and SD for lysis was calculated for each treatment (Figure 2B). The control containing VBS/BSA and anti-CD59 showed minimal lysis. Marked increase in neuronal cell lysis was observed on exposing the mixed central nervous system (CNS) cells for 30 minutes to NHS (diluted 1/8) containing the anti-CD59 neutralizing antibody. The cells treated with NHS diluted 1/8 containing anti-CD59 antibody and sCR1 showed a significant reduction in the overall numbers of lysed neurons.

Figure 6. Detection of MCP and CD59 mRNAs expressed by human fetal brain mixed-cell culture after ISH. ISH was performed using digoxigenin-UTP labeled riboprobes. The end product was visualized by immunoalkaline phosphatase/NBT/BCIP resulting in staining of positive sites. **a:** MCP sense riboprobe. Neurons (**short arrows**) and astrocytes (**long arrows**) were not stained. Original magnification, $\times 190$. **b:** MCP anti-sense probe. Neurons (**short arrows**) are stained specifically as well as astrocytes (**long arrows**). Original magnification, $\times 190$. **c:** CD59 sense riboprobe. Neurons (**short arrows**) and astrocytes (**long arrows**) were not stained. Original magnification, $\times 100$. **d** and **e:** CD59 anti-sense probe. Neurons (**short arrow**) and astrocytes (**long arrows**) were stained. Original magnifications, $\times 100$ and $\times 190$, respectively.

Double-Immunostaining to Analyze the Expression of Complement Regulatory Proteins by Human Fetal Neurons and Astrocytes in Culture

DAB immunoperoxidase staining (anti-GFAP, brown staining) together with BCIP/NBT immunoalkaline phosphatase staining (anti-CD56 or anti-complement regula-

tory proteins, blue staining) were performed to analyze the expression of complement regulators by human fetal brain cells. The results are shown in Figure 3. Human fetal neurons and astrocytes were negative for OX23 (an irrelevant mAb to factor H) (Figure 3a, short arrowhead). Neurons were positive for CD56 as indicated by the blue staining of the plasma membrane (Figure 3b, short ar-

rowhead). Neurons were negative for CR1 (Figure 3c, arrowhead) and DAF (Figure 3d, arrowhead) as indicated by the absence of blue staining, and MCP immunostaining was very weak (Figure 3e, arrowhead). Faint blue immunostaining with mouse anti-CD59 was consistently observed on neurons (Figure 3f, short arrow).

Astrocytes, as expected were strongly positive for the specific cytoskeletal marker GFAP (brown staining) (Figure 3, a–f). No blue staining of astrocytes was detected with OX23 (Figure 3a, long arrow). Blue staining of astrocytes with a mAb to CD56 was consistently observed (Figure 3b, long arrow). No staining was detected on astrocytes using the rabbit anti-CR1 (Figure 3c, long arrow). Strong staining was observed with the rabbit anti-DAF (Figure 3d, long arrow). The co-localization of the brown (DAB) staining from anti-GFAP and the blue (NBT/BCIP) product from anti-DAF resulted in a dark brown staining of the astrocytes (Figure 3d, long arrow). Strong immunostaining with anti-MCP was detected on astrocytes (Figure 3e, long arrow) whereas a more delicate membrane staining of astrocytes with anti-CD59 was observed (Figure 3f, long arrow).

Flow Cytometric Analysis to Confirm the Differential Expression of Complement Regulatory Proteins on Mixed Human Fetal Brain Cultures

Mixed fetal brain cultures were stained for complement regulators by indirect immunofluorescence and the level of staining was assessed by flow cytometry (Figure 4). The mean level of fluorescence (FL2 channel) of the small-size population (neurons; confirmed by NSE staining) and the large-size cell population (astrocytes; confirmed by GFAP staining) depicted from the cell scatter plot (forward scatter *versus* side scatter) was used to assess the relative abundance of each complement regulator on the cell membranes of each cell type. The results are presented in Figure 4 and the mean level of the fluorescence intensity (FL2) for each immunostain is given in brackets. CD56 and CD44 immunostaining were included as positive controls. Neurons expressed higher levels of CD56 (FL2: 393) and low levels of CD44 (FL2: 59) (Figure 4, 1a). Astrocytes expressed high levels of CD56 (FL2: 1723) and CD44 (FL2: 3102) (Figure 4, 1b). CR1 was not detected either on neurons (Figure 4, 2a) or on astrocytes (Figure 4, 2b), whereas DAF was absent on neurons but expressed by astrocytes (FL2: 38), albeit at a low level (Figure 4; 3, a and b). MCP was weakly expressed by neurons (FL2: 22) and astrocytes (FL2: 120) (Figure 4; 4, a and b). All cells expressed CD59; the staining was weak on neurons (FL2: 420) and strong on astrocytes (FL2: 3534) (Figure 4; 5, a and b).

Constitutive Expression of Complement Regulator mRNAs from Human Fetal Brain Cultures: RT-PCR Analysis

RT-PCR results from human fetal brain mixed cultures demonstrated an abundant expression of MCP and CD59

mRNAs, whereas DAF mRNA was weakly expressed (Figure 5). In contrast, we consistently found no CR1 mRNA expression in any of four samples of human fetal brain cultures (Figure 5). RT-PCR of GAPDH mRNA and clusterin, a fluid phase complement regulator known to be expressed in brain,^{44–46} were used as internal positive controls (Figure 5).

Expression in Situ of Complement Regulator mRNAs by Human Fetal Brain Cells in Culture

ISH for MCP and CD59 was performed using digoxigenin-UTP-labeled riboprobes on human mixed fetal brain primary cultures. The MCP and CD59 sense riboprobes were consistently negative (Figure 6, a and c) but the antisense riboprobes for MCP and CD59 were positive. The ISH signal from the antisense MCP probe was weak in neurons (Figure 6b, short arrow) and stronger in astrocytes (Figure 6b, long arrows). In contrast, CD59 antisense riboprobe localized strongly to neurons (Figure 6d, short arrows) and to astrocytes (Figure 6, d and e, long arrows). The GAPDH antisense probe was positive for neurons and astrocytes with the same intensity of staining on both cell types (not shown).

Discussion

The brain is an immunologically isolated site protected by a blood brain barrier that restricts infiltration of circulating pathogens from blood to neuronal tissue.⁴⁷ We and others have proposed that recruited and activated glial cells (astrocytes and microglia) can produce locally, innate immune molecules to recognize and kill pathogens which may infiltrate the CNS through a damaged blood brain barrier.^{23,24,38,39,48–50} The complement system is an important component of the innate immune defense mechanism and activated glial cells have the capacity to generate a complete and functional complement system with cytotoxic and cytolytic activities.^{21,22,24,33,50–52} Complement may also have adverse effects. For instance uncontrolled activation of the complement system in the CNS has been associated with a number of pathologies including multiple sclerosis, Alzheimer's disease, Pick's disease, and Huntington's disease.^{9–18} The mechanisms by which human neurons activate the complement system are poorly understood.

Nucleated cells such as endothelial cells, epithelial cells, and even hepatocytes (the major cellular source of complement) are consistently exposed to complement which can be spontaneously activated on their membrane by the tick-over mechanism.^{53–55} Although activation of complement on the cells could lead to lysis, it is now well known that the majority of nucleated cells control complement activation and complement-mediated lysis by expressing specific complement regulators. Published studies have documented complement activation and regulator expression by diverse cell lines of apparent neuronal origin, the majority of which are susceptible to complement killing.^{33–35} In one neuronal cell line, a cru-

cial role for CD59 in protecting from complement killing has been described.^{35,56} Despite these data, little is known about the capacity of human primary neurons *in vitro* to control complement activation by expressing complement regulators.

We undertook a systematic investigation *in vitro* to test whether human neurons cultured from fetal brain could spontaneously activate the complement system, and to assess the capacity of neurons to express the complement regulators CR1, MCP, DAF, and CD59. We found that fetal neurons but not astrocytes were opsonized and lysed by complement via activation of the classical pathway when cultured in the presence of NHS. Importantly, serum devoid of immunoglobulins opsonized the cells to a similar degree, effectively eliminating an important role of antibody in the observed activation. No complement opsonization was detected when the cells were incubated with heat inactivated NHS or NHS containing EDTA. No factor B staining was detected on the neurons treated with NHS or immunoglobulin-deficient NHS, indicating that the alternative pathway was not involved in the activation process. The data suggest that neurons express a molecule that can specifically bind C1q and initiate classical pathway activation. The identity of the neuronal C1q receptor remains to be characterized. It has been hypothesized that in disease conditions, C1q could bind to molecules such as DNA, mitochondrial membranes, myelin, and amyloid released in the vicinity of the neurons.^{19,20,57,58} However, it is unlikely that these molecules released from damaged cells are the factors involved in the spontaneous activation of complement on cultured fetal neurons. Of note, adjacent astrocytes were not opsonized to a significant degree, indicating that the activation was occurring specifically on the membranes of fetal neurons.

It is clear that human fetal neurons were not able to control complement activation because we observed that human fetal neurons were lysed in the presence of NHS on neutralization of CD59 activity. Astrocytes were not lysed by complement when exposed to the same conditions. Human fetal neurons were unable to prevent complement activation because they expressed low levels of MCP and lacked DAF and CR1; this has been confirmed by a number of techniques in this investigation. Characterization of the expression of membrane-bound complement regulators by human fetal brain primary cultures at the mRNA level using RT-PCR showed a complete absence of CR1, confirming the negative staining of cells at the protein level both by immunocytochemistry and fluorescence-activated cell sorting analysis. MCP is reported to be predominantly a regulator of the alternative pathway.^{59–61} Absence of DAF and poor classical pathway regulation by MCP on neurons would facilitate effective C3b and C5b-9 deposition. Astrocytes were found to express MCP, DAF, and CD59 at higher levels than neurons and hence were not lysed even though they lacked CR1. Soluble CR1 inhibited lysis of human fetal neurons confirming that complement was responsible for lysis. As CR1 is a regulator of the C3/C5 convertases and has

been shown to be a potent inhibitor of complement-mediated lysis in CNS disorders,^{62,63} it represents a potentially valuable neuroprotective agent.

This *in vitro* study demonstrates that neurons are extremely susceptible to complement activation and complement-mediated lysis, although the mechanism responsible for neuronal complement activation is not clear. It will be important in future studies to identify the receptor expressed specifically by neurons and not by astrocytes that is able to bind C1q and trigger complement activation. This could be addressed by performing crosslinking experiments using ¹²⁵I-labeled C1q. We have previously shown that two human neuroblastoma cell lines (IMR32 and SKNSH) expressing low levels of MCP and CD59 and lacking DAF and CR1 activated the classical pathway and were lysed in the presence of NHS.³³ The results obtained using the primary neuron cultures corroborate the data from these neuroblastoma cell lines, suggesting that they could be used to identify the putative neuronal C1q receptor involved in the complement activation process. It has been reported that oligodendrocytes are also susceptible to complement-mediated lysis because they not only activate spontaneously the classical pathway of complement but also express low levels of regulators.^{28,32} Adult human oligodendrocytes in culture were found to express low levels of DAF and CD59 and were lacking CR1 and MCP.²⁸ Together, these data suggest that there is a deficit in regulation of the classical pathway of complement activation at the C3/C5 convertase stage by both neurons and oligodendrocytes. A recent *in vitro* study⁶⁴ has demonstrated that a human neural crest-derived cell line (Paju) expressed increased levels of DAF after PMA (phorbol ester) treatment. It remains important to assess in future studies whether neurons exposed to proinflammatory stimuli (cytokines) or even after sublethal complement attack can be induced to express increased levels of complement regulators to protect themselves from further complement attack.

We have recently shown that neurons *in vivo* also lack DAF and CR1,⁶⁵ providing a direct correlation of the expression of complement regulators between our *in vitro* culture model and adult neurons *in vivo*. Manipulation of the endogenous levels of regulatory proteins on these cells would be of potential importance in the development of therapy to block the resulting pathology after local complement activation. It has been shown that sCR1 is a potential therapeutic agent in experimental animal models to prevent demyelination and in stroke.^{62,63} It will be informative to expand this study to use sCR1 and other complement regulators such as DAF in animal models of neurodegeneration.

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