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Complement Factor H, a Marker of Self Protects against Experimental Autoimmune Encephalomyelitis[1]

Mark R. Griffiths,* Jim W. Neal,† Marc Fontaine,‡ Trina Das,§ and Philippe Gasque[2]*§

The CNS innate immune response is a "double-edged sword" representing a fine balance between protective antipathogen responses and detrimental neurocytotoxic effects. Hence, it is important to identify the key regulatory mechanisms involved in the control of CNS innate immunity and which could be harnessed to explore novel therapeutic avenues. In analogy to the newly described neuroimmune regulatory proteins also known as "don't eat me" signals (CD200, CD47, CD22, fractalkine, semaphorins), we herein identify the key role of complement regulator factor H (fH) in controlling neuroinflammation initiated in an acute mouse model of Ab-dependent experimental autoimmune encephalomyelitis. Mouse fH was found to be abundantly expressed by primary cultured neurons and neuronal cell lines (N1E115 and Neuro2a) at a level comparable to BV2 microglia and CLTT astrocytes. Mouse neurons expressed other complement regulators crry and low levels of CD55. In the brain, the expression of fH was localized to neuronal bodies and axons, endothelial cells, microglia but not oligodendrocytes and myelin sheaths and was dramatically reduced in inflammatory experimental autoimmune encephalomyelitis settings. When exogenous human fH was administered to disease Ab-dependent experimental autoimmune encephalomyelitis animals, there was a significant decrease in clinical score, inflammation, and demyelination, as compared with PBS-injected animals. We found that the accumulation of human fH in the brain parenchyma protected neurons from complement opsonization, axonal injury, and leukocyte infiltration. Our data argue for a key regulatory activity of fH in neuroprotection and provide novel therapeutic avenues for CNS chronic inflammatory diseases. ***The Journal of Immunology,*** **2009, 182: 4368–4377.**

Classically, innate immune cells such as neutrophils, NK cells, dendritic cells, and macrophages are involved in the selective recognition and the clearance of pathogens and toxic cell debris during infection or tissue injury (1–3). However, there is little evidence of an immunosurveillance of the brain by these peripheral cells and it is now evident that resident cells, glial cells, ependymal cells, and neurons are capable of mounting a robust innate immune response on their own (4–10). This response is based upon the recognition of "non-self" and "altered-self" patterns, also called "danger signals," by molecules and receptors expressed essentially by microglia but also found on astrocytes, oligodendrocytes, and neurons (11, 12). These molecules and receptors are called pattern recognition receptors and are released in soluble forms or displayed on the cell membrane of the phagocyte. Whereas much attention has been focused on the properties and activities of the TLRs in this process (13), many other innate immune molecules expressed by glia and neurons have been described (e.g., complement, lectins, scavenger receptors) (14).

Critically, uncontrolled innate immune responses can contribute to cytotoxic and cytolytic activities and must be polarized to avoid neuronal loss and robust neuroinflammation as reported in demyelinating and neurodegenerative diseases (12, 15–21).

Macrophages and microglia can kill and phagocytose target neuronal cells and it is equally important to understand the mechanisms controlling their eager appetite (11, 22). Key regulatory mechanisms of innate immunity have recently been described and thought to be at the root of neuroprotective mechanisms. For instance, there is new emphasis on the role of neuroimmune regulatory proteins (NIRegs)[3] that are involved in silencing or reshaping an adverse innate immune response and polarizing phagocytes such as macrophages and microglia toward a protective phenotype (for review, see Ref. 23). NIRegs were originally termed self-associated molecular patterns on the grounds that they are expressed by self-viable cells to control the inflammatory reaction at the site of injury (11, 24). Several NIRegs expressed by neurons have now been described such as CD47, CD46, CD200, CD22, fractalkine, and semaphorins, which behave as "don't eat me" or "repulsive" signals to prevent unwarranted phagocytosis (12, 25–30).

Human fetal neurons and neuronal cell lines have the capacity to regulate complement pathway activation by expressing several of

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[3] Abbreviations used in this paper: NIReg, neuroimmune regulatory protein; DAF, decay-accelerating factor; MS, multiple sclerosis; fH, factor H; MOG, myelin oligodendrocyte glycoprotein; ADEA, Ab-dependent experimental autoimmune encephalomyelitis; EAE, experimental autoimmune encephalomyelitis; LFB/CV, Luxol fast blue/cresyl violet; TRITC, tetramethylrhodamine; DAPI, 4′,6-diamidino-2-phenylindole; DAB, diaminobenzidine; NFL, neurofiilament L chain; TCS, tissue culture supernatant.

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the key regulators including C1 inhibitor, membrane cofactor protein (CD46), and CD59 but fail to express decay-accelerating factor (DAF; CD55) (31–34). Critically, it remains to be tested whether this relative protection is functional in situ and particularly in disease conditions associated with high levels of complement activation due either to a disruption of the blood-brain barrier or to an increased local biosynthesis (35). In mice, the expression of the CR1-related protein y (crry) in the brain has been reported and contributes to the regulation of both the classical and alternative complement pathways (36). The cytopathic activity of complement has been linked to neurodegeneration and neuroinflammation on the grounds that neurons and oligodendrocytes have a propensity to activate complement by mechanisms that remain ill-characterized (34). In animal models of multiple sclerosis (MS), it has been demonstrated that the complement alternative pathway is a potent route to demyelination and neurodegeneration (37). Of further note, genetic polymorphisms of factor H (fH), a regulator of the complement alternative pathway, are associated with several degenerative disorders, including age-related macular degeneration. In contrast, a protective role of complement regulator fH has been observed recently against complement-mediated killing of human glioblastoma cells (38).

In this study, we explored the expression by neurons of the alternative pathway C regulators such as fH and tested whether fH could provide the CNS with protection against complement-mediated inflammation, axonal injury, neuronal loss, and demyelination in a mouse model of acute MS, the myelin oligodendrocyte glycoprotein (MOG)-induced Ab-dependent experimental autoimmune encephalomyelitis (ADEAE). We investigated the potential therapeutic effect of fH on the severity of the clinical and pathological changes in mice with ADEAE.

Materials and Methods

Animals

C57BL/6 mice (Harlan) were maintained in specific pathogen-free conditions. All animal procedures were in accordance with institutional and Home Office guidelines. Ten- to 12-wk-old mice were used in all experiments, with 10 mice in each experimental condition. Tissues from $fH^{-/-}$ mice were obtained from Dr. M. Botto (Imperial, London, U.K.).

RT-PCR

Expression of the complement regulators of the C3 convertases, i.e., factor H, crry, and CD55 were tested by RT-PCR as previously described (39). Total RNA was extracted from the brain and spinal cord, and cDNA was obtained by transcription with Moloney murine leukemia virus reverse transcriptase and the PCR fragments were amplified with the Platinum *Pfx* polymerase (Invitrogen). The fH PCR product was a 489-bp sequence taken from the National Center for Biotechnology Information Entrez Nucleotide database, reference BC066092, comprising the region 3177–3665 bp. The fH forward primer was 5′-CCACATGTGCCAAATGCTAC-3′ and the reverse primer was 5′-AAATGGCGGTGAATCTCTTG-3′. The crry forward primer was 5′-GGATCTAGACGTTGTTTGAGAAC-3′ and the reverse primer was 5′-GTTACTAGTGAAGCAGCTTGGTA-3′, amplifying a product size of 900 bp. The CD55 product was 151 bp, amplifying a product in exon 1 of the gene between bases 83 and 233. The CD55 forward primer was 5′-TCAATTAACTGCGGCTCAAA-3′ and the reverse primer was 5′-GGACAGCAGCAACAGAGACA-3′. The GAPDH primers were as described to amplify a product of 473 bp (39).

Mouse complement regulation by human fH

Highly purified human fH was obtained by hydrophobic affinity chromatography essentially as described previously (40). The purity of the preparation was confirmed by HPLC (Proteomelab; Beckman Coulter) and SDS-PAGE and with the identification of a unique 140-kDa protein as expected. Serum from a naive mouse was collected by exsanguination and centrifugation. Zymosan A (10 mg/ml) (Sigma-Aldrich) was suspended in 0.15 M NaCl and boiled for 1 h before centrifugation and resuspension in PBS with either EGTA (10 mM) and $MgCl_2$ (5 mM) or EDTA (10 mM) as negative control. One volume of 10 mg/ml zymosan suspension was mixed with 1 volume of 5% serum with EGTA/$MgCl_2$ and the equivalent of 0, 125, 250, or 500 μg/ml human fH in PBS. The zymosan and serum were incubated at 37°C for 1 h before washing in PBS-BSA. C3 opsonization of the zymosan particles was examined by labeling with anti-C3c Ab (DakoCytomation A0062), which is cross-reactive for mouse C3. Detection of primary Ab was with donkey anti-rabbit R-PE (Jackson ImmunoResearch Laboratories). Mean fluorescence was measured by FACS analysis as described elsewhere (39).

Role of fH in the control of neuroinflammation and demyelination

On day 0, experimental autoimmune encephalomyelitis (EAE) was induced in C57BL/6 mice following s.c. immunization at a single site close to the base of the tail with 200 μl of an emulsion consisting of a 1:1 mixture of CFA and PBS containing 2.5 mg/ml *Mycobacterium tuberculosis* H37 Ra and 0.5 mg/ml recombinant mouse MOG consisting of aa 1–117 of the extracellular Ig domain (rMOG; provided by Dr. H. Reid (Monash University, Clayton, Victoria, Australia) (19). CFA and *M. tuberculosis* H37 Ra (MtbH37) were from Difco (Epsom). Mice also received 200 ng of pertussis toxin i.p. (Sigma-Aldrich) in PBS on days 0 and 2.

The same procedure was followed in a separate group of C57BL/6 mice as for the induction of the EAE until day 9, when each animal in this group received an i.p. injection of Z12 mAb (0.5 mg in PBS) to induce ADEAE (19). The anti-MOG mAb Z12 was produced as previously described (41). Subsequently, one group of ADEAE mice were injected i.p. with purified human fH (0.5 ml at 2 mg/ml); the other group with an equal volume of sterile PBS. We did not use BSA as a carrier and negative control to test for fH activity given that high levels of albumin are already present in the mouse serum.

For both EAE and ADEAE groups, mice were weighed daily and monitored for signs of clinical disease which was scored as follows: 0, no disease; 1, tail atony; 2, hind limb weakness; 3, hind limb paralysis; 4, moribund; and 5, dead. Animals that attained a clinical score of 3 or 4 at monitoring were sacrificed immediately to conform to Home Office guidelines.

Tissue processing and analysis

EAE and ADEAE animals were sacrificed while under terminal anesthesia and the brains and spinal cords were removed. The brain was cut sagittally to divide the two hemispheres and the spinal cord was quartered approximating to the cervical, thoracic, lumbar, and sacral regions. One-half of the brain and the cervical and lumbar spinal cord sections were immediately frozen in isopentane on dry ice. These tissues were cryostat sectioned at 10 μm. The remaining CNS tissue was postfixed in 4% paraformaldehyde in PBS for paraffin wax embedding and microtome sectioning. Analysis and quantitation of inflammation and demyelination was performed blind by a neuropathologist observer (J.W.N.) essentially as described previously (19).

Paraffin sections (6-μm thick) were cut and stained with H&E for analysis of inflammation. Inflammatory cells in subpial and perivascular areas were separately scored on arbitrary scales of 0–4. For subpial inflammation, scoring was as follows: 0, no infiltration into the subpial space; +, minor infiltration; 2+, numerous infiltrating cells; 3+, most of the pia involved; and 4+, all of the pia involved. For perivascular inflammation, scoring was as follows: 0, no perivascular inflammatory cells; +, one or two cuffed vessels per section; 2+, three to five cuffed vessels per section; 3+, five to eight cuffed vessels; and 4+, more than eight cuffed vessels per section. Demyelination was assessed for each experimental group in Luxol fast blue/cresyl violet (LFB/CV) stained sections; the area in mm^2 of myelin loss and total white matter area were measured using a graticule and the percentage demyelination was reported on a 4-point scale from 0 to 4 for complete loss of myelin. (0, no demyelination; 2+, perivascular demyelination; 4+, extensive perivascular and subpial demyelination with formation of confluent plaques), essentially as published (19).

Immunohistochemical analysis of complement regulators and markers of neuroinflammation

For immunofluorescence, fresh frozen sections were left to air dry for 1 h before fixation in ice-cold acetone stored at −20°C for 5 min. Sections were then allowed to dry before marking with a pap pen and washing in PBS and blocking in PBS-BSA for 30 min. Sections were then incubated with the relevant primary Ab or isotype control for 1 h at room temperature using the manufacturer's recommended dilutions, before further repeated washes and blocking, and then incubated in secondary Ab which was either

Table I. *FACS analysis of fH expression in mouse model cell lines of neurons, glial cells, macrophages, and endothelial cells: comparison with other innate immune regulators and cell surface markers*[a]

Innate Regulators	N1E115 Neuron	Neuro2A, Neuron	CLTT, Astrocyte	BV2, Microglia	RAW264.7, Macrophage	Bend3, Endothelial
mAb anti-Crry	165	101	185	108	133	123
mAb anti-CD55	7	5	129	71	279	285
mAb anti-CD59	120	56	25	34	43	97
Rb[b] anti-fH	591	202	483	319	1584	431
Rb anti-CD14	Neg	Neg	Neg	68	134	30
Rb anti-CD141	36	17	26	136	279	44
mAb anti-CD31	Neg	Neg	Neg	15	73	1424
mAb anti-CD24	314	169	408	32	129	Neg
mAb anti-CD43	Neg	Neg	Neg	Neg	933	Neg
mAb anti-CD44	Neg	251	2696	2128	Neg	856
mAb anti-CD91	51	82	115	128	50	42
mAb anti-CD18	Neg	Neg	Neg	89	282	Neg
mAb anti-CD97	Neg	Neg	neg	80	203	Neg

[a] Mean of FL2 signal above background, $n = 2$.
[b] Rb, Rabbit; Neg, negative.

conjugated to FITC or tetramethylrhodamine isothiocyanate at a 1/200 dilution (Jackson Immunoresearch Laboratories). Sections were simultaneously incubated with 4′,6-diamidino-2-phenylindole (DAPI) at 0.1 μg/ml to stain nuclei in blue. Following an overnight incubation, sections were washed and mounted with Vectorshield (H-1000; Vector Laboratories). For the detection of the mouse complement regulators, rabbit anti-rat CD55 and anti-crry Abs made in house using highly purified proteins were used and were cross-reactive for mice, as confirmed by Western blotting (see Fig. 2 and Ref. 42). The rabbit polyclonal anti-mouse fH was raised in house using fH-Ig recombinant protein and comprising mouse fH (SCR1–4) fused to human IgG4 (gift from Dr. C. L. Harris, Cardiff University, Cardiff, U.K.). This Ab was immunoabsorbed on a human total IgG column to eliminate reactivity against Igs. We confirmed that this Ab detected mouse fH but not Ig in mouse serum while no reactivity was observed in $fH^{-/-}$ mouse serum (provided by Dr. M. Botto, Imperial). The affinity-purified rabbit anti-human fH was described previously (8). For double immunofluorescent stainings, we used the rat anti-mouse NeuN (MAB 377; Chemicon International.) to detect neurons and the rat anti-CD31 (clone 390; BD Pharmingen) to stain endothelial cells in vessels. Infiltrating macrophages were stained for CD68 (clone FA-11; Serotec).

To quantify neurofilament L chain protein and C3 deposition on cells in the gray matter, sections were stained using the immunoperoxidase diaminobenzidine (DAB) protocol (34). Initially, sections were fixed in acetone and allowed to air dry before blocking endogenous peroxidase activity by incubating in 0.3% H_2O_2 for 5 min. Endogenous biotin was then blocked using the Vector Laboratories avidin/biotin blocking kit (SP2001) according to the manufacturer's instructions. Sections were then blocked in PBS-BSA for 30 min and incubated with the primary Abs for 1 h at room temperature. After repeated washes in PBS, sections were incubated in the appropriate biotinylated secondary Ab, washed, and then complexed with avidin-biotin (Vectorstain Elite ABC kit PK-7200; Vector Laboratories). DAB was then used as chromogen following the instructions in the Vector DAB kit (SK-4100). C3 deposition (DakoCytomation A0062) was calculated as relative area using the Openlab software program (19). For neurofilament L chain staining (AHP286; Serotec), the reaction incorporated DAB-Ni^{2+} for increased sensitivity. After allowing the staining to develop, the reaction was stopped in PBS. Sections were then dehydrated through an increasing gradient of ethanol before mounting with surgipath mounting medium and coverslipping for microscopic analysis. Neurofilament L chain (NFL)-positive labeled neurons were then counted within the gray matter. NFL is a structural protein evenly distributed within cells but was shown to aggregate in the neuronal body and axons of injured neurons. High levels of NFL in the spinal cord of EAE animals can be used to reliably quantify the amount of neuronal damage (43).

Immunohistochemical detection of fH on cultured neurons

Primary cultures of mouse E19 neurons were prepared by dissociation of brains in HBSS and filtration through a 70-μm pore. Cells were harvested by centrifugation and resuspended in neurobasal medium supplemented with B27 under FCS-free conditions (Invitrogen). Neurons were grown on poly-L-lysine-coated sterile coverslips and the medium was changed on days 4 and 8. On day 9, excess medium was removed from the coverslips before a 10-s fixation in ice-cold acetone. Double Immunofluorescent cytochemistry was then performed on cells as detailed above using a mouse anti-NeuN Ab to stain neurons specifically. Primary cultures also contained NeuN-negative cells which were essentially $GFAP^+$ astrocyte cells.

fH expression was also analyzed for the mouse neuroblastoma cell lines Neuro2a (N2a) and N1E115 and compared with that of the mouse macrophage cell line (RAW264.7) and mouse brain endothelial cell line (Bend3) all obtained from the European Collection of Cell Cultures. The mouse microglia (BV2) and mouse astrocyte (CLTT) cell lines were obtained from Dr. M. Mallat (Institut National de la Santé et de la Recherche Médicale Unité 495, Paris, France). The human neuroblastoma cell line KELLY was from the ECACC. Cells were grown on coverslips in DMEM with 10% FCS before fixation in acetone for immunostaining. The expression of membrane-bound cell markers before or after stimulation with PMA or cytokines was ascertained by FACS analysis as previously described (39). We expressed the data as mean intensity of the fluorescence (FL2) above background staining using an irrelevant primary Ab. Cells were stained using rat anti-mouse Abs against crry (clone 5D5; in house), CD55 (2C6; in house), CD59 (rat Mel4; in house), CD31 (390; BD Pharmingen), CD24 (M1/69; BD Pharmingen), CD43 (BD Pharmingen), CD44 (5D2/27; Developmental Studies Hybridoma Bank (DSHB)), CD91 (2C6; DSHB), CD18 (M182A; DSHB), and anti-CD97 (F4/80; Serotec). DSHB Abs were obtained from the Department of Biological Sciences, University of Iowa (Iowa City, IA). Rabbit Abs were used to stain for fH (see above), CD14 (Santa Cruz Biotechnology/Autogen Bioclear), and CD141 (Santa Cruz Biotechnology) (see Table I).

Western blotting and ELISA

To ascertain whether proinflammatory stimuli can modulate the expression of fH, human and mouse neuron cell lines were treated for 24 h with either phorbol ester (PMA) to induce cell differentiation or recombinant proinflammatory cytokines IFN-γ, IL-1β, or TNF-α provided by Hoffmann-La Roche. Other recombinant cytokines and growth factors were purchased from PeproTech and used from 5 to 200 ng/ml (see Fig. 1 legend). Cell lines were grown until 80% confluent in T25 flasks (10^6 cells) and then stimulated. After a 6-h incubation, cell tissue culture supernatant (TCS; 5 ml) was harvested and dialyzed overnight in distilled water to remove salts. Dialysates were then frozen at −80°C and lyophilized using a freeze drier as described elsewhere (8). The remaining dry powder was resuspended in 200 μl of Laemmli buffer. The same cytokine stimulation regimen was conducted in 24-well plates (1×10^5 cells/200 μl) and the TCS was collected to measure the level of secreted fH by ELISA essentially as described previously (6, 8). In brief, we used the rabbit anti-mouse fH for the coating and the same biotinylated Ab for the detection using streptavidin peroxidase. For human fH, the mouse monoclonal OX24 was used for the coating and the biotinylated polyclonal anti-human fH (740) for the detection.

Whole cell lysates were also prepared in PBS containing 2% Nonidet P-40 along with protease inhibitors as previously described (8). All samples diluted in nonreducing Laemmli buffer were warmed to 37°C before loading 15 μl into each well of a 4–12% Nu-PAGE gradient precasted gel (Invitrogen). After transferring the gels onto nitrocellulose membranes and

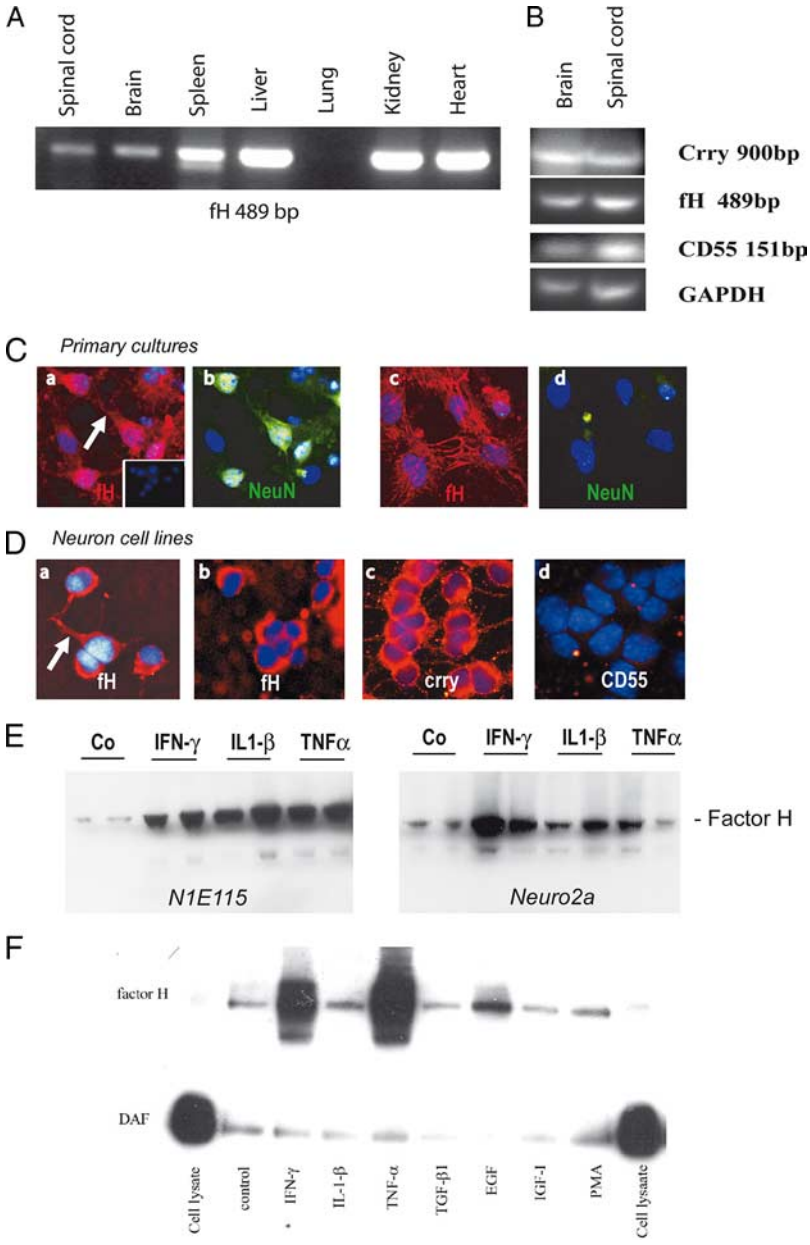

FIGURE 1. Neurons express abundant levels of membrane-bound and soluble fH, particularly in response to proinflammatory cytokines and growth factors. *A* and *B*, Neuronal tissue samples were taken from a naive C57BL/6J mouse, RNA was extracted, and cDNA was obtained by RT-PCR for three major C3 regulators, i.e., fH, crry, and CD55. GAPDH was used as a housekeeping gene. *C*, Expression of fH in mouse primary neuronal cells of wild-type mice. $NeuN^+$ (neuron) and $NeuN^-$ (astrocyte) cells were strongly stained for fH (rabbit anti-mouse fH). The membrane fH staining was localized to the cell body and to axons (arrows). (*Ca*, *inset*), Background staining was observed on primary neurons from $fH^{-/-}$ mice. Nuclei were lightly counterstained in blue with DAPI. Original magnification, ×200 (*D*, *a* and *b*). The mouse neuroblastoma cell lines N1E115 (*a*) and Neuro2a (*b*) were strongly immunostained for fH. *Da*, A prominent fH staining was evidenced on the axons (arrow). Neuro2a was also immunostained with mAbs for crry (*Dc*) but showed very weak immunostaining for CD55 (*Dd*) (original magnification, ×200). CD55 and crry were not expressed in primary neuronal E19 cultures (data not shown). *E*, Stimulation of N1E115 and N2a induced strong expression of fH in cell culture supernatants. Cells were not stimulated (two individual controls) or stimulated with IFN-γ (200 ng/ml and 20 ng/ml from *left* to *right*), IL-1β (20 and 2 ng/ml), and TNF-α (50 and 5 ng/ml) over 24 h. Rabbit anti-mouse fH was used. *F*, The stimulation of the human neuronal cell line Kelly with cytokines and growth factors increases fH expression as shown by Western blot analyses. CD55 was detected mainly in cell lysates, in contrast to fH solely detected in TCS. Cells were stimulated with IFN-γ, IL-1β, TNF-α, TGF-β1, epidermal growth factor(EGF), insulin growth factor 1 (IGF-1), and PMA. Rabbit anti-human fH was used.

blocking in PBS milk, membranes were incubated overnight at 4°C with the affinity-purified anti-human fH and anti-human CD55 Abs (1/1000), in the case of Kelly. For N1E115 and N2a, the rabbit anti-mouse fH Ab was used (1/4000). Following further washes, gels were incubated with goat anti-rabbit secondary Ab (1/4000; Bio-Rad) before ECL (Pierce) detection. Prestained broad range protein markers from New England Biolabs were used as molecular mass standards.

Statistical analysis

All comparisons of nonparametric data (clinical score and demyelination) were performed using Mann-Whitney *U* nonparametric tests; the exact two-tailed *p* value corrected for ties is quoted throughout. The Student *t* test was applied to parametric data. Two-tailed *p* values are quoted throughout. ANOVA was performed for C3 opsonization of zymosan particles and a two-sample *t* test was used for post hoc analysis.

Results

Expression of complement regulators in mouse tissues

We first ascertained the relative expression of fH in brain tissues when compared with other tissues. Although fH RNA was detected in the brain, the highest level of expression was observed in kidney, heart, liver, and spleen, whereas the lungs surprisingly failed to express detectable levels of fH (Fig. 1*A*). Naive mouse spinal cord and brain tissue were tested for expression of several complement regulators following RT-PCR. crry, fH, and CD55 (DAF) cDNA were detected for both CNS tissues (Fig. 1*B*).

fH expression in mouse primary neuronal cultures and neuronal cell lines

Mouse E19 primary neuronal cultures were prepared on poly-L-lysine-coated coverslips. Immunohistochemical staining using highly specific Abs revealed the presence of fH on NeuN-positive primary neuronal cells (Fig. 1*Ca*). No staining was observed on $fH^{-/-}$ mouse brain tissue sections (data not shown). The cell body and the axonal projection were strongly stained for fH (white arrow) with a dual distribution at the cell surface and in the cytoplasm. NeuN-negative cells (Fig. 1*Cc*) were also strongly stained for fH and are more likely to be the feeder layer of astrocyte cells also stained for the glial acidic fibrillary protein (GFAP) (data not shown). FACS analysis performed on viable mouse E19 neuronal

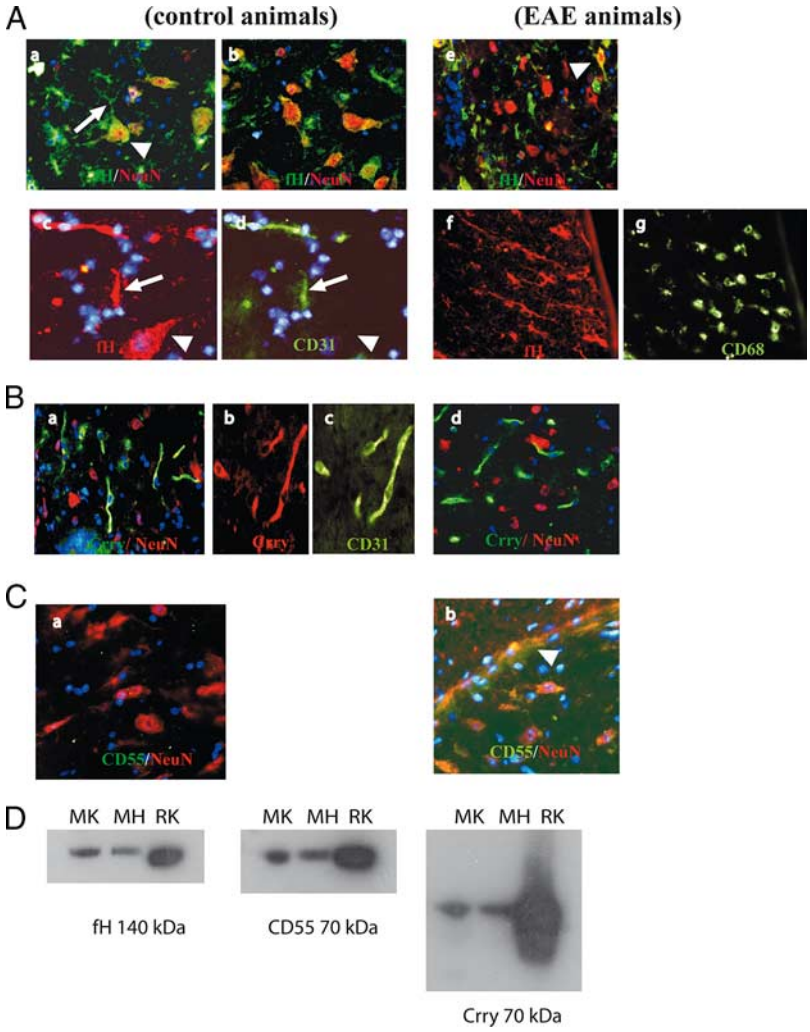

FIGURE 2. Neurons express a restricted set of C3 complement inhibitors in naive and in inflamed conditions. We analyzed the expression of the complement regulators fH, CD55, and crry control and in EAE animals. *A*, Expression of fH (green staining, rabbit anti-mouse fH) at the cell body (arrowhead) and axons (arrow) was evidenced on NeuN-positive neurons (red) in mice without disease (*a* and *b*). Robust fH staining was also localized to vessels as indicated by the costaining for fH and CD31$^+$ endothelial cells (*c* and *d*, arrow). Neurons stained for fH are negative for CD31 (arrowhead). In mice with EAE disease (clinical score 3), the expression of fH was dramatically lost on neurons (*e*) but was prominent on CD68-positive macrophage cells (green) and radial glial cells in the white matter (*f* and *g*) and gray matter (data not shown). Very few neurons remained positive for fH in EAE-inflamed conditions (*Ae*, arrowhead). *B*, Staining for crry (green, rabbit) is not associated with NeuN-positive neurons (red) in mice without (*a–c*) or with (*d*) clinical disease. Crry is however strongly detected on CD31-positive blood vessels (green; *b* and *c*). *C*, DAF (CD55, green, rabbit Ab) expression was not detected in mice without disease (*a*, NeuN in red) but was weakly detected on NeuN-positive neurons in those mice with disease (*b*). CD55 was also detected within the extracellular matrix of the glia limitans, an area of active leukocyte infiltration (*Cb*) *D*, Western blot detection of mouse fH, CD55, and crry by the polyclonal Ab anti-rat inhibitors to confirm cross-reactivity and specificity. MK, Mouse kidney; MH, mouse heart; RK, rat kidney.

cells further indicated that fH was present at the cell membrane and with a mean fluorescence intensity of 156 ± 48 ($n = 4$, $p = 0.003$, FL2 channel) above background staining using irrelevant Abs. The unique cellular distribution pattern of fH was confirmed by immunostaining of the mouse neuroblastoma cell line N1E115 and, in particular, with an axonal distribution of fH (Fig. 1*Da*, white arrow). N2a was strongly labeled for fH and crry but very poorly for CD55 (Fig. 1*D*, *b–d*, respectively). These immunostaining results were confirmed by FACS analysis (Table I) to detect fH expression and other neuronal and glial markers. Both neuronal cell lines expressed high levels of fH and crry but CD55 was only weakly detected at the cell membrane. In contrast, the other mouse brain cell lines of astrocyte, microglia, macrophage, and endothelial origins expressed high levels of all C3 regulators (fH, crry, CD55) and CD59 (Table I). The phenotype of the different cell lines was confirmed by immunostaining for CD14 (microglia, macrophage), CD31 and CD141 (endothelial cells and macrophages), CD43 (macrophages), CD44 (astrocyte and microglia but not macrophage), and CD24 (particularly expressed by neurones and astrocytes) (Table I) (44–49).

Cytokines and growth factors modulate fH expression by neuronal cells in vitro

Human and mouse neuronal cell lines were grown in vitro and examined for their fH response following stimulation with a panel of cytokines, growth factors or PMA (to induce cell differentiation). The mouse cell lines N1E115 and N2a did alter expression of fH following stimulation and particularly in response to all proinflammatory cytokines (Fig. 1*E*). Furthermore, the human neuronal cell line Kelly did massively up-regulate fH expression following stimulation with either of the Th1 cytokines IFN-γ or TNF-α, but not IL-1. Stimulation with the cytokines or PMA did not result in an increase in soluble CD55 expression, serving as an internal control (Fig. 1*F*). However, CD55 was detected in Kelly cell lysates. The level of fH produced over 24 h by mouse (N1E115) and human (Kelly) neuronal cell lines was further tested by ELISA of TCS ($n = 3$). N1E115 expressed, respectively (4.5 ± 3.2 ng/ml/5 × 10^5 cells; 152 ± 77 ng/ml/5 × 10^5, $p < 0.05$; 216 ± 50 ng/ml/5 × 10^5, $p < 0.05$; and 201 ± 23 ng/ml/5 × 10^5, $p < 0.05$), in control conditions and after stimulation with 200 ng/ml IFN-γ, 20 ng/ml IL1-β, and 50 ng/ml TNF-α. For Kelly cells, we found that they expressed constitutively (8 ± 3 ng/ml/5 × 10^5 cells and 409 ± 150 ng/ml/5 × 10^5, $p < 0.05$) in response to 20IU/ml human IFN-γ over 24 h.

Neurons in the CNS express fH but not the other membrane-bound C3 complement regulators

Spinal cords were removed from naive mice and mice with EAE. Immunohistochemistry was performed on sections from these mice. Animals were sacrificed at day 15. Sections were colabeled with the neuronal marker NeuN and C3 complement regulators, namely, crry, CD55, or fH. fH expression was found extensively on the neurons of naive mice, localized to both the cell body (arrowhead) and axons (arrow) (Fig. 2*A*, *a* and *b*).

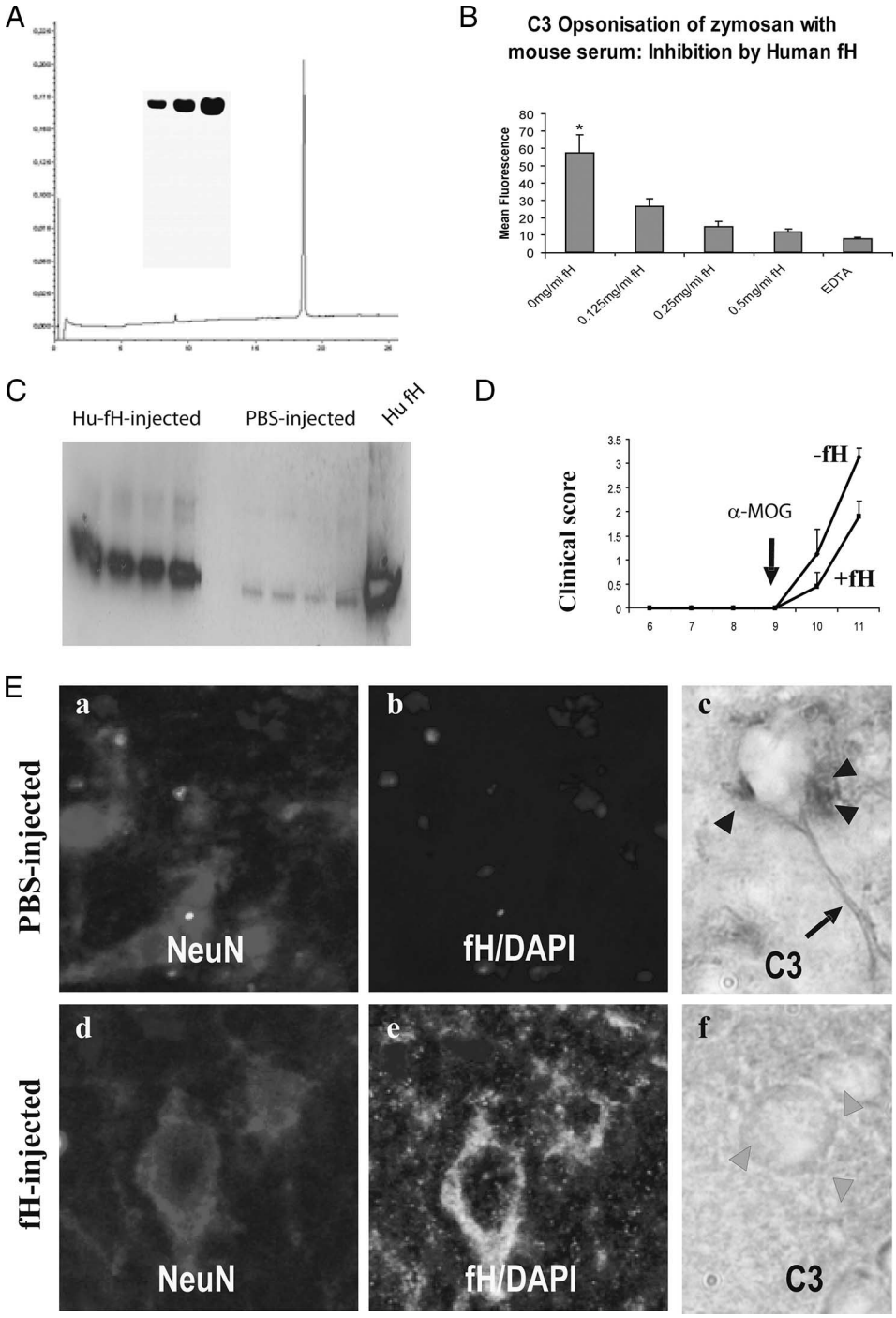

FIGURE 3. A single delivery of human fH to the brain protects from clinical neuroinflammation and complement activation and in a mouse model of acute ADEAE. *A*, The purity of the human fH was confirmed by HPLC (Abs, *y*-axis; fraction number, *x*-axis) and by SDS-PAGE analyses of 2, 4, and 8 μg of the purified protein (*inset*). *B*, We next tested the functional inhibition of mouse C3 opsonization using highly purified human fH. Opsonization of zymosan by mouse C3 was inhibited by the addition of human fH at 125, 250, and 500 μg/ml. Controls included EDTA treatment to block complement activation cascade. *C* and *D*, Mice were randomly assigned to receive an injection of either sterile PBS or human fH (i.p.) on day 9 following MOG injection to establish EAE. These mice were simultaneously injected with anti-MOG Ab (Z12, 0.8 mg/ml, i.p.) to induce acute Ab-dependent demyelination with a consistent disruption of the blood-brain barrier (data not shown). *C*, Human fH was detected in the serum of mice ($n = 4$) 48 h after a single i.p. injection. In PBS-injected mice, no human fH was detected and there was very weak cross-reactivity with mouse fH. We used the affinity-purified rabbit anti-human Ab for the Western blottting. This Ab has only a weak reactivity against mouse fH. *D*, Clinical disease was recorded for both PBS and human fH-injected mice ($n = 10$/group). Disease severity was reduced and delayed in fH-injected mice but progressed very quickly in PBS-injected mice, and all animals were sacrificed by day 11 according to the Home Office's regulations. *E*, Human fH located in the spinal cord was strongly associated to $NeuN^+$ neurons (*E*, *d* and *e*) and also to extracellular matrix around blood vessels (data not shown). DAPI was used to counterstain nuclei. There was a strong C3 opsonization (dark staining) close to neuronal body (black arrowhead) and axons (arrow) in PBS-injected ADEAE mice in contrast to human fH-injected ADEAE mice (gray arrowhead; *f*); original magnification, ×400.

However, fH labeling was considerably reduced on the neurons of disease EAE mice (Fig. 2*Ae*). The mean fH staining intensity (taken from four individual fields of view) was dramatically reduced in EAE mice when compared with control mice (186.2 ± 95.9 vs 847.5 ± 410 U respectively, t = 2.1, $p = 0.04$). Only rarely did neurons remain fH positive (arrowhead Fig. 2*Ac*). fH also colocalized with CD31-positive blood vessels in naive mice (Fig. 2*A*, *c* and *d*, arrow). Neurons stained for fH were CD31 negative (Fig. 2*A*, *c* and *d*, arrowhead). In the inflammatory EAE setting, fH expression was strongly up-regulated on CD68-labeled microglia and with radial glial cells (Fig. 2*A*, *f* and *g*).

Crry expression did not appear to colocalize with neurons in naive or EAE-induced mice (Fig. 2*B*, *a* and *d*). However, crry was expressed on blood vessels in the CNS as demonstrated by colocalization with CD31 (Fig. 2*B*, *b* and *c*). In EAE, crry staining remained restricted to endothelial cells (Fig. 2*Bd*). In naive mice, CD55 expression was extremely weak or below detectable limits by fluorescence microscopy (Fig. 2*Ca*). However, CD55 expression was detected on neurons in EAE-induced mice (Fig. 2*Cb*) and also within the extracellular matrix at the level of the glia limitans of the spinal cord (Fig. 2*Cb*, arrowhead). Although the rabbit polyclonal Abs for crry and CD55 were raised against rat Ags, we validated that they also recognized the corresponding mouse proteins. Western blot analysis using both mouse kidney and heart, or rat kidney, revealed specific labeling of fH at 140 kDa, and CD55 and crry at 70 kDa (Fig. 2*D*).

Table II. *Measurement of inflammation in the spinal cord of PBS and human fH-injected ADEAE mice*

Identity	Leukocyte Infiltration				Demyelination	Clinical Score
	Meninges	Perivascular	White matter	Gray matter		
PBS	+++	+	++/+++	−	++	3.13 ± 0.18
Human fH	+	−/+	+	−	+	1.89 ± 0.3

Scoring of inflammatory infiltrating cells ranges from − (not detected) to +++ (high levels of infiltration, see *Materials and Methods* for scoring).

Regulation of mouse complement alternative pathway by human fH

Activated zymosan was incubated along with mouse serum to cause deposition of mouse C3 onto the zymosan particles via the alternative pathway. The zymosan was incubated either in the absence of human fH or with the equivalent of 125, 250, or 500 μg/ml human fH in PBS. In the absence of human fH, there was a mean fluorescence of 57.3 (Fig. 3*B*). By comparison, when human fH was added in increasing concentration, mean fluorescence was 26.62, 14.87, and 11.63, respectively. When serum and zymosan were incubated together in the presence of EDTA, mean fluorescence read 7.9. ANOVA showed that there was a large significant difference when all groups were analyzed ($f = 13.92$, $p < 0.0002$). Post hoc analysis by *t* test comparison between the serum-only condition and those with human fH showed that at each concentration used, human fH significantly reduced mouse C3 opsonization (Fig. 3*B*).

Down-regulation of ADEAE pathology in mice following therapeutic administration of human fH

Before onset of disease in the mice, all animals were injected with Z12, a mouse anti-MOG mAb, to induce a reliable ADEAE disease. This remains a valuable model of MS that drives severe pathology associated with disruption of the blood-brain barrier to allow the transudation of complement and exogenous complement regulators tested herein within the brain parenchyma. The mice ($n = 20$) were randomly assigned to two groups, one to receive an i.p. injection of PBS and the other group to receive an i.p. injection of human fH. Human fH was successfully incorporated into the circulatory system and persisted for 48 h, the duration of the study after injection (Fig. 3*C*). ADEAE is an acute form of EAE in which 100% of the animals showed clinical signs of disease within 48 h following injection with the anti-MOG Ab. In this study, 100% of the mice coinjected with PBS showed clinical signs of disease and 90% coinjected with human fH reached clinical disease. The cumulative disease index for the human fH-injected mice was 21.5 and 49.5 for the PBS control group. Mann-Whitney *U* analysis demonstrated that mice in the human fH-injected group had a significantly lower disease score than those in the PBS-injected group on day 11 (Mann-Whitney *U* test = 65, $p = 0.006$; Fig. 3*D*). The level of leukocyte infiltration in several areas of the spinal cord and the incidence of demyelination was dramatically reduced in fH-injected ADEAE animals (Table II).

Immunohistochemical analysis demonstrated human fH immunolabeled cells in the spinal cord of fH-injected mice (Fig. 3*Ed*). In the white matter where there was infiltration, human fH was detected (data not shown). NeuN-labeled neurons in the spinal cord gray matter were also labeled for human fH (Fig. 3*Ed*). PBS-injected mice showed no immunoreactivity for human fH using the affinity-purified polyclonal Ab (Fig. 3*Eb*). Inflammation and demyelination were measured by a blinded neuropathologist observer. For all parameters measured, PBS-injected mice had an overall higher level of inflammation and demyelination (Table II).

Complement deposition was also examined by assessing the level of C3 opsonization in spinal cord sections and the relative area for C3 deposition was calculated by image analysis. Mice injected with human fH had a lower level of C3 deposition in the gray matter compared with the PBS-injected mice (214.4 ± 45.9 and 368.5 ± 40.8 U, respectively, $t = 2.5$; $p = 0.046$). Fig. 3*Ee* depicts the distribution of C3 staining on neurons, axons, and cell body in PBS-injected ADEAE animals. In sharp contrast,

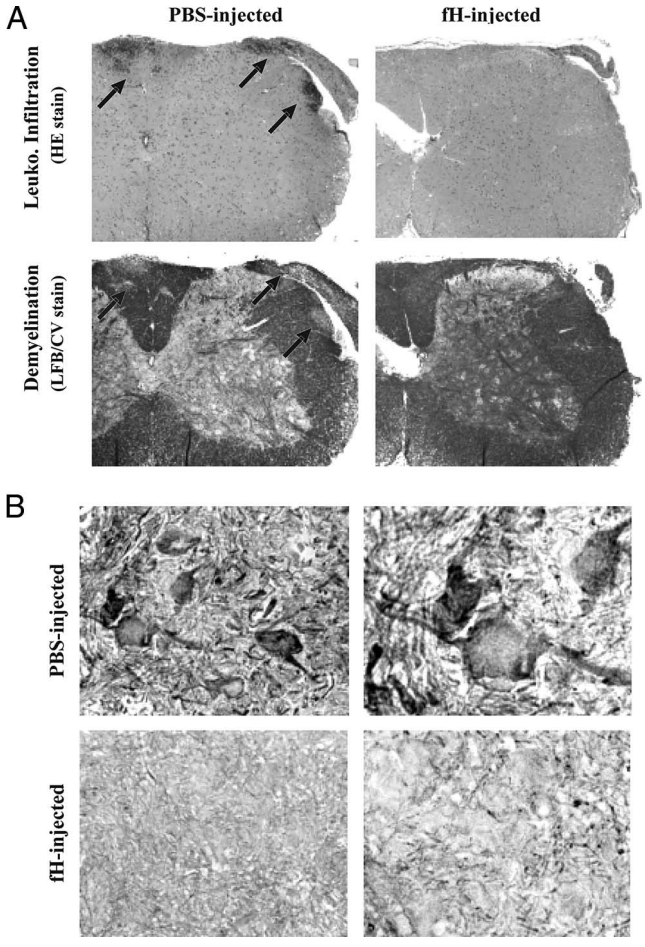

FIGURE 4. Therapeutic application of a single bolus of human fH protects mice from neuroinflammation and neuronal/axonal injuries. The level of leukocyte infiltration and associated demyelination (*A*) was compared between PBS-injected and fH-injected mice using H&E (*top*) and LFB/CV (*bottom*), respectively). Serial sections clearly illustrated that acute demyelination was seen in areas of severe inflammation and leukocyte infiltration in ADEAE mice (arrows). A single fH i.p. injection was able to confer protection from neuroinflammation and demyelination (*right panels*). *B*, Overall level of neuronal and axonal injuries: Neuronal bodies stained strongly for NFL, a marker of neuronal and axonal injuries, and there was an increased number of NFL-positive cells in PBS vs fH-injected mice. Two magnifications are presented (×200 and ×400).

much less C3 opsonization was detected in the spinal cord of fH-injected ADEAE mice (Fig. 3*Ef*, gray arrowhead).

The level of neuroinflammation, namely, demyelination associated with active leukocyte infiltration and neuronal damage was assessed using H&E and LFB/CV stainings as well as labeling for NFL expression in the gray matter of spinal cord sections from ADEAE mice injected with either PBS or human fH (Fig. 4). There was a more robust inflammatory infiltration in the meninges and white matter of the spinal cord correlating with severe demyelination in PBS-treated animals (Fig. 4*A, left panels*, arrows) when compared with fH-injected animals. This acute neuroinflammation in the white matter was also correlated with an increased number of NFL-positive labeled cells indicative of severe neuronal injury in mice injected with PBS compared with human fH-injected mice at the time of Z12 administration (46.01 ± 7.51 and 20.6 ± 4.42 respectively, $t = 2.92$, $p = 0.02$).

Discussion

A number of studies have reported the protective effects that membrane-bound and fluid-phase complement regulators have in models of autoimmunity including EAE (19, 34, 36, 50–53). fH is an important regulator of the alternative complement pathway because it has cofactor activity with factor I, dissociating factor B from C3b, and also controlling the C3 and C5 convertases by interacting with C3b. There is good reason therefore to investigate the regulation of the alternative pathway in complement-mediated models of MOG-induced EAE because C3 and factor B-deficient mice have attenuated EAE (54). Importantly, fH has been implicated in a number of systemic diseases, including age-related macular degeneration, hemolytic uremic syndrome, Alzheimer's disease, and membranoproliferative glomerulonephritis type II, associated with an inflammatory component (55–58).

Despite these observations, the contribution fH makes regulating the innate immune system and more specifically the alternative C activation in mouse EAE was unknown. In this study, in vitro analysis with PCR and immunohistochemistry demonstrated expression of fH in CNS mouse spinal cord neurons and in both E19 mouse neuronal and mouse neuroblastoma cells. The relevance of human fH as a regulator of C in mice was confirmed using FACS analysis that demonstrated a concentration-dependant decrease in mouse C3b deposition, indicating that human fH is capable of inhibiting components of the mouse alternative C pathway. Furthermore, stimulation of cells in vitro with proinflammatory cytokines and epidermal growth factor increased fH in the supernatant, supporting previous findings (33). Interestingly, we found that fH was secreted in the cell culture supernatant but was also associated with the cell membrane as described for monocyte U937 cells (59).

After induction of EAE as a model of neuroinflammation and following disease onset, fH immunolabeling was surprisingly lost from the spinal cord neurons, whereas CD55 expression was notably elevated. Furthermore, we found neurons to be crry negative. At this time, crry and fH expression was detected on $CD31^+$ endothelial cells. fH appears to be lost from neurons under inflammatory conditions in EAE, perhaps as the result of negative regulation of a factor that remains to be characterized. In contrast, our in vitro data argue for a stimulatory effect of classical proinflammatory cytokines in the stimulation of fH expression. It is also possible that fH is synthesized by neurons, rapidly secreted but not retained at the surface of dying neurons as a consequence of the disease process. However, the localization of mouse fH with CD68-positive macrophages and CD31 endothelial cells provides an ideal cellular location to inhibit the alternative C pathway. A further set of experiments using exogenously administered human fH was therefore conducted to investigate whether or not the addition of exogenous fH could be delivered to neurons and to promote therapeutic effect on acute CNS inflammation and neuronal injury.

The Ab-mediated demyelinating form of EAE, ADEAE, is an aggressive model of MS that has a well-established complement component driving disease (37, 53). This model is also characterized by a disruption of the blood-brain barrier and hence allowing for the delivery of therapeutic components to the brain parenchyma. When exogenous human fH was administered to these ADEAE mice, there was a significant decrease in clinical score, inflammation, and demyelination, as compared with the PBS-injected group. Protein analysis confirmed that human fH was present in serum 48 h after i.p. injection in the treated animals, the duration of their survival. Neurons within the spinal cord of the human fH-treated group were stained for human fH. Exogenous fH enters the CNS from the systemic circulation and within 48 h is sequestered onto cell membranes, providing protection against C attack. Neuronal damage was assessed by NFL staining in the soma of cells in the gray matter. NFL aggregates in the neuronal soma during axonal injury (60) and NFL has been reported to aggregate in the neuronal body and axons in the spinal cord of rats with EAE (43). In this study, NFL aggregates were also found in spinal cord sections of mice with EAE. NFL was found to be significantly elevated in mice spinal cord sections from the PBS-injected group, compared with the human fH-injected mice. This protection was characterized by a lower level of NFL labeling of neurons in the gray matter of mice in the human fH-injected group. This lower level of NFL in the latter group was correlated with a lower clinical disease score, supporting the idea that fH has important neuroimmune regulatory activity in the inflamed CNS. It will be important to validate this observation with fH-deficient mice but with the complicated issue that they are also depleted in C3 through rapid turnover (61).

The neuroprotection offered by the exogenous fH might well be through its actions as a regulator of complement activation. Several tumor cell lines inhibit complement-mediated lysis through expression of proteins that sequester fH to the tumor cell surface, thereby inhibiting C lysis (62). Similarly, glioblastoma cells express fH to increase cleavage of C3b to iC3b. This protection was not similarly afforded by the membrane-bound complement regulators CD46, CD55, and CD59 (38). Immunohistochemical analysis of tissue sections from the two groups of mice revealed a decrease in spinal cord area stained with C3 in the human fH-injected mice. The decrease in C3 deposition on neuronal cells in the spinal cords of these mice may be critical in the less severe pathology observed. This result is supported by the finding that C3-deficient mice had attenuated EAE (54).

Our data support the idea that fH is an important neuroimmune regulatory protein. These are a group of molecular signals that distinguish host from non-self or altered self (pathogens and apoptotic cells) (11). The presence of fH will prevent targeting by the innate immune system, whereas its absence will allow attack notably by complement expressed by activated microglia-expressing C3 receptors involved in phagocytosis. It is reported that some pathogens bind fH to escape detection and prevent opsonization with complement and in this instance fH acts as a subverted self-regulatory protein to confer protection (63, 64). fH was also found on blood vessels and macrophages in an inflammatory setting, possibly to protect from complement attack. Interestingly, a cell membrane receptor for fH has been reported on phagocytes (65, 66) which could be involved in cell-cell interactions between neurons expressing fH at the cell membrane and macrophages carrying the receptor. Experiments are highly warranted to further characterize

the fH receptor and ascertain its signaling properties. In rats, fH has also been identified as an adrenomedullin-binding protein and this interaction enhances the complement regulatory activities of fH (67, 68). Adrenomedullin expression in rat cortex has a cytoprotective effect in a model of ischemia-reperfusion injury (69, 70). Although adrenomedullin expression has not been investigated in this study, it is plausible that the fH and adrenomedullin may interact to limit disease severity in EAE.

In summary, this study has shown that neurons have a limited expression of C3 regulators restricted mostly to fH expression. Furthermore, we found that fH was decreased on neurons in severely inflamed CNS tissues. Acute administration of human fH resulted in sequestration of fH on neurons and provided protection from complement attack resulting in attenuated EAE-induced disease and reduced levels of inflammation. This modulation of disease may have been through a direct regulation of complement activation alone or in concert with an alternative mechanism whereby fH inhibitory activity is enhanced through interacting proteins such as adrenomedullin or through a fH receptor on macrophages. This alternative should be further investigated because it may prove advantageous in the development of future therapeutics that inhibits acute CNS inflammation.

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Disclosures

The authors have no financial conflict of interest.

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