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Short Communication

Expression of the Receptor for Complement C5a (CD88) Is Up-regulated on Reactive Astrocytes, Microglia, and Endothelial Cells in the Inflamed Human Central Nervous System

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C5a receptor (C5aR, CD88) is a receptor originally described on neutrophils and monocyte-macrophages but recently found on hepatocytes, epithelial cells, endothelial cells, and tissue mast cells. We recently reported that human fetal astrocytes expressed a functional C5aR in vitro. Here we examine C5aR expression in adult brain cultures by immunostaining with six different anti-C5aRs and show that C5aR is expressed constitutively by astrocytes, microglia, and fibroblast-like cells but not by oligodendrocytes. In fetal brain cultures we confirmed that astrocytes constitutively expressed C5aR and demonstrated that fetal microglia and fibroblast-like cells but not oligodendrocytes and neurones expressed C5aR. Incubation with inflammatory cytokines (interferon γ, interleukin-1, and tumor necrosis factor α) or phorbol ester failed to induce or up-regulate C5aR expression on fetal or adult brain cells. Immunohistochemistry was performed to determine the expression and distribution of C5aR in the normal and inflamed brain. In the normal brain C5aR was minimally expressed, whereas in inflamed brains from a variety of pathologies, C5aR expression was greatly up-regulated on reactive astrocytes and microglia and to a lesser extent on endothelial cells. We propose that expression of C5aR is a marker of central nervous system inflammation, and that C5aR expression on brain cells in inflammation plays an important role in cell activation and recruitment (gliosis). (Am J Pathol 1997, 150:31–41)

C5a anaphylatoxin is a chemoattractant molecule released locally in tissues at sites of complement activation. Neutrophils, eosinophils, and monocyte-macrophages express a receptor for C5a (C5aR, CD88),[1–5] and these cells will migrate from the peripheral site to the inflammatory site in response to a gradient of C5a. In addition to recruiting cells, C5a is also a powerful stimulator of leukocytes, inducing expression of proinflammatory substances (eg, cytokines).[5–11] Expression of the C5aR has recently been demonstrated on human hepatocytes, epithelial cells, endothelial cells, and tissue mast cells.[12–15] We have reported the presence of a functional C5aR on human fetal astrocytes[16] and have suggested that generation of C5a has an important role in cell recruitment and activation in the brain.

Complement activation has been demonstrated in numerous inflammatory and degenerative diseases

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of the brain, including multiple sclerosis (MS) and Alzheimer's disease (for review see articles by Morgan[17] and Morgan and Gasque[18]). We wish to ascertain whether products of complement activation mediate cell infiltration into the disease foci and activation or damage to resident and infiltrating cells. Here we report that the C5aR is expressed on cultured astrocytes and microglia derived from adult and fetal brains. Expression of C5aR *in situ* in the normal brain was very low, but in areas of inflammation associated with a variety of pathologies, including demyelination, infection, and neurodegeneration, C5aR expression was greatly up-regulated on reactive astrocytes and microglia and to a lesser extent on endothelia.

Materials and Methods

Antibodies

All mouse monoclonal anti-C5aR clones (S5/1, W17/1, D12/1, and P12/1) have been described previously.[4] Two rabbit polyclonal anti-C5aR peptide antibodies (9–30 and 1–30) were generated in our laboratory. The first antibody was generated by immunization with a C5aR peptide (amino acids 9–30) coupled through an amino-terminal KA (lysine-alanine) peptide linker to keyhole limpet hemocyanin, as described previously.[16] The second antibody was generated by immunization with a multiple-array (MAP; Applied Biosystems, Foster City, CA) C5aR peptide (amino acids 1–30). The specificity of both anti-C5aR peptides was confirmed by fluorescence-activated cell sorting analysis and Western blot on differentiated THP1 cells.

Other monoclonal antibodies (MAbs) used in this study were mouse anti-glial fibrillary acidic protein (GFAP) (clone GA5, 1:1000; Sigma Chemical Co., Poole, UK), mouse anti-galactocerebroside (GC, 1:1000; Ranscht et al[19]), mouse anti–neuron-specific enolase (NSE) (clone BBS/NC/VI-H14, 1:2000; Dako, Bucks, UK), mouse anti-CD11b (clone 2LPM19c, 1:50; Dako), mouse anti-CD68 (1:50, clones KP1, PG-M1, and EBM11; Dako), and mouse anti-human leukocyte antigen (HLA) class II (clone LN3, 1:10 to 1:50; Biotest, Solihull, UK). Polyclonal antibodies (PAbs) anti-GFAP (1:1000; a generous gift of Dr J. Newcombe, MS Laboratories, London, UK), anti-GC (Sigma), and antineurofilament (NF) 200 (Sigma) were used for double-staining experiments. Rabbit anti-proteolipid protein was raised in house (by Dr S. Piddlesden, Medical Biochemistry Department, University of Wales College of Medicine, Cardiff, UK) and used for immunostaining of oligodendrocytes and myelin in formalin-fixed tissue sections. Mouse anti-τ (clone Snl 51, 1:1500; Affinity Research Products, Derbyshire, UK) was used to detect Pick's bodies in Pick's disease (PD) brains as described previously.[20]

Cell Culture and Immunocytochemistry

Primary cultures of human fetal glial cells and neurons were established from fetal brains (6- to 10-week fetuses) obtained from the Medical Research Council Tissue Bank (Hammersmith Hospital, London, UK) as described previously.[16,21] Primary cultures (passage 0, days 1 to 7) contained clusters of neurons (demonstrated by immunostaining with an antibody against NF70, NF200, or neuron-specific enolase; see Figure 2d) lying on a monolayer of cells. Some 60 to 80% of the cells in this monocellular layer expressed the astrocytic marker GFAP (see Figure 2b), 5 to 7% were microglia (immunostained with three different anti-CD68 antibodies), and 15 to 40% were fibroblasts (GFAP-negative cells identified by their morphology). Fetal brain cultures at passage 0 also contained a very low percentage of oligodendrocytes (<1%), which were identified by immunostaining using an antibody against GC (see Figure 2c). Microglia were removed by changing the medium at days 2 and 4 and by orbital shaking (350 rpm, 1 hour, and 37°C at day 7). After shaking, cultures were changed to fresh medium and left in 5% CO_2/95% O_2 at 37°C for at least 2 hours. Neurons and oligodendrocytes were removed from the astrocyte monolayer at day 7 by an additional orbital shaking (250 rpm overnight at 37°C). Adherent cells were trypsinized and subcultured the following day. Fibroblasts were removed by preplating the cell suspension in a culture flask for 1 hour at 37°C to enrich the cell suspension for astrocytes. Experiments using fetal astrocytes were conducted on cells from culture passages 1 to 3.

Normal adult temporal lobe tissue was obtained fresh from biopsies of patients (two cases) undergoing therapeutic resection for intractable epilepsy. These were used for culture of glial cells, as described by Armstrong et al.[22] The cell suspension obtained after mechanical and enzymatic treatment was placed on 13-mm glass coverslips precoated with 10 μg/ml poly-L-lysine. After incubating for 1 hour at 37°C, the medium was changed, and cells were cultured on coverslips for 14 days in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum, antibiotics, and L-glutamine. The medium was changed every 2 days, and at day 14 cells were cultured for 2 days in

fetal bovine serum-free medium. $GFAP^+$ elongated astrocytes and $CD11b^+$, $CD68^+$ microglia-macrophages were identified by immunostaining and represented the two major populations in the adult brain cultures. GC^+ oligodendrocytes were also occasionally present in adult brain cultures, whereas no NSE^+ neurons were detected. $GFAP^-$, GC^-, and $CD11b^-$ cells with fibroblast-like morphology were also detected in adult brain cultures.

The human promonocytic cell line THP1 was used as a positive control for C5aR staining as described previously.[16] In some experiments, the THP1 line was differentiated to its macrophage phenotype using phorbol ester (50 ng/ml for 3 days) to up-regulate expression of C5aR, CD11b, and CD68. THP1 cells with or without differentiation remained negative for NSE, GFAP, and GC.

For staining, cells cultured on sterile poly-L-lysine-coated glass coverslips were washed three times in phosphate-buffered saline (PBS), fixed, and immunostained according to a protocol described previously in detail.[16] Cells were either fixed with 4% formaldehyde (membrane immunostaining) or were fixed and permeabilized with 95% ethanol/5% acetic acid (membrane and cytoplasmic staining). All primary antibodies were diluted in PBS/1% serum albumin and applied overnight at 4°C. After extensive washing, cells were incubated with different secondary antibodies: alkaline phosphatase (AP)-conjugated anti-immunoglobulin (Ig) with 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium substrate to give blue staining, according to the manufacturer's instructions (Sigma); and horseradish peroxidase (HRP)-conjugated anti-Ig with diaminobenzidine (DAB) substrate to give brown staining (Sigma); fluorescein isothiocyanate-conjugated anti-Ig (goat anti-rabbit, Sera-Lab, Crawley Down, Sussex, UK; rabbit anti-mouse, Dako) was used for indirect immunofluorescence staining, and fluorescence was visualized either on an inverted fluorescence microscope (Nikon Inc, Melville, NY) or a confocal laser scanning microscope (TCS; Leica, Wetzlar, FRG). For confocal laser scanning microscopy, 12 optical sections were collected per field at 0.3-μm intervals from the bottom to the top of the cell, and sections were assembled as extended-focus views. For double immunostaining, adherent cells were fixed with ethanol/acetic acid, washed, and incubated overnight at 4°C with both a polyclonal cell-specific antibody and monoclonal anti-C5aR. Rabbit polyclonal anti-GFAP, anti-NF200, and anti-GC (all diluted 1:1000) were used to identify respectively astrocytes, neurons, and oligodendrocytes.

All anti-C5aR MAbs were tested; clones W17/1 and P12/1, which gave a strong, specific staining at very low concentration were used in most studies. Clones S5/1 and D12/1 anti-C5aR stained fixed, permeabilized cells very poorly. After incubation in primary antibody, coverslips were extensively washed and incubated overnight at 4°C with both fluorescein isothiocyanate-conjugated donkey anti-mouse Ig and rhodamine-conjugated goat anti-rabbit Ig (Jackson ImmunoResearch Laboratories, West Grove, PA). After washing, coverslips were mounted on slides with Fluorsave mounting medium (Calbiochem, Nottingham, UK). Fluorescence was imaged as described above.

No polyclonal marker specific for microglia was available; therefore, double staining for microglia was carried out using monoclonal anti-CD68 and polyclonal anti-C5aR peptide-(1–30).

Source of Tissue and Processing

Brain tissue was obtained locally at postmortem or from specialist tissue collections. Tissue was collected from individuals with a variety of brain disorders (demyelination, neurodegeneration, and viral infection) and normal control subjects. Tissue samples from four cases of MS (three acute plaques and one chronic plaque) were obtained from Dr Jia Newcombe (MS Society Laboratory, London, UK), and tissue samples from six cases of PD with Pick inclusion bodies were obtained from Dr Nigel J. Cairns (Medical Research Council Alzheimer's Disease Brain Bank, Institute of Psychiatry, London, UK). Tissue was obtained locally from individuals with Huntington's disease (HD) (16 cases), human immunodeficiency virus (HIV) encephalitis (two cases) and non-HIV viral encephalitis (two cases). Control brain tissue was obtained at postmortem from individuals with no evidence of neurodegenerative disease or ischemia and was of a similar age and postmortem interval (maximum, 30 hours) profile to the disease samples. Brains were cut coronally, and individual blocks from areas of the brains containing macroscopic evidence of pathology were dissected. Tissue was either snap frozen and kept at −40°C or fixed in 10% formalin before processing for cryosections or paraffin wax embedding and sectioning, respectively.

Light Microscopy and Immunochemistry

Rehydrated paraffin sections were counterstained with hematoxylin and eosin (H&E) to display morphology; PD brain sections were instead counterstained with cresyl violet to identify Pick bodies.[20]

Table 1. *Immunocytochemistry of Human Monocyte Cell Line (THP1, PMA differentiated), Adult and Fetal Brain Cultures*

		Fetal brain cultures			Adult brain cultures		
Antibody (clone)	THP1/PMA	Astrocyte	Neurons	Oligodendrocyte	Astrocyte	Oligodendrocyte	Microglia
MAb anti-GFAP	−	+++	−	−	++++	−	−
MAb anti-GC	ND*	−	−	+++	−	+++	−
MAb anti-NSE	ND	−	++	−	ND	ND	ND
MAb anti-CD68	+++	−	−	−	−	−	++
MAb anti-CD11b	+++	−	−	−	−	−	+
MAb anti-HLA-Dr	++	+/−	−	−	+	−	+
MAb anti-C5aR (S5/1)	++	+	−	−	+	−	++
MAb anti-C5aR (W17/1)	++++	+++	−	−	++	−	++
MAb anti-C5aR (D12/1)	++	+	−	−	+	−	++
MAb anti-C5aR (P12/1)	+++	++	−	−	++	−	++
				−			
PAb anti-GFAP	−	++	−	−	−	−	−
PAb anti-GC	ND	−	−	+++	−	+++	−
PAb anti-NF200	ND	−	++	−	+/−	−	−
PAb anti-C5aR peptide 1-30	+++	++	−	−	+++	−	−

THP1 was differentiated for 3 days with phorbol ester (PMA) before immunostaining.
* ND, not determined.

Luxol fast blue (LFB) stain was used to identify demyelinating plaque areas in MS tissue.

Rehydrated paraffin wax sections and, when available, cryosections (8 μm) from normal and diseased brains were immunostained with antisera or MAbs to the C5a receptor, all antibodies were diluted in PBS/bovine serum albumin and detected using an indirect immuno-HRP/DAB method as described previously.[20] Swine anti-rabbit Ig-HRP and rabbit anti-mouse Ig-HRP (1:100 dilution; Dako) were used as secondary antibodies.

Results

Immunostaining of Fetal and Adult Brain Cultures for C5aR

Four well-characterized anti-human C5aR MAbs (S5/1, W17/1, D12/1, and P12/1) and two polyclonal anti-human C5aR peptides (9–30 and 1–30) were used in this study. All were generated against the N-terminal part of the C5a-binding site of the receptor (amino acids 1 to 30). Optimal dilutions of antibodies was determined by immunostaining of phorbol 12-myristate 13-acetate (PMA)-differentiated THP1 cells followed by fluorescence-activated cell sorting analysis. MAbs were used at 0.1 μg/ml, and polyclonal antisera were used at a 1:1000 dilution (Table 1). None of the antibodies gave detectable staining of the C5aR-negative human B lymphocyte cell line. Figure 1 shows the immunofluorescence staining of THP1 cells using W17/1 anti-C5aR, before differentiation (Figure 1a) and after PMA differentiation for 3 days (Figure 1b) to induce a macrophage-like phenotype. All undifferentiated THP1 cells were stained for C5aR, but only a small number of cells (1 to 5%) expressed the receptor at a high level (one cell in the center of the field). After PMA stimulation all THP1 cells (now firmly adherent with small processes) abundantly expressed C5aR (Figure 1b). The same results were obtained with the other anti-C5aR reagents. Up-regulation of C5aR expression correlated with the up-regulation of CD11b and CD68 expression on differentiated THP1 cells (Table 1).

All antibodies were then tested on human fetal brain cultures (five samples) and human adult brain cultures (two samples) using indirect immunofluorescence staining. Adult brain cultures (passage 0) were ethanol/acetic acid fixed and immunostained. The cultures contained elongated astrocytes ($GFAP^+$), fibroblast-like cells ($GFAP^-$, $CD68^-$), microglia-macrophages ($CD11b^+$, $CD68^+$) and a small number of oligodendrocytes (GC^+) (Table 1). A typical filamentous immunostaining of astrocytes was observed using monoclonal anti-GFAP (Figure 1c), whereas patchy membrane staining was observed using W17/1 anti-C5aR (Figure 1d). Double immunostaining was carried out using a polyclonal anti–cell-specific marker and monoclonal W17/1 anti-C5aR. All $GFAP^+$ astrocytes were also $C5aR^+$. The same result was obtained using different clones of anti-C5aR (Table 1). The staining obtained with S5/1 and D12/1 was weak because of the ethanol/acetic acid fixation protocol. Double immunostaining using anti-CD68 and polyclonal anti-C5aR-(1–30) confirmed that $CD68^+$ microglia were also $C5aR^+$ (Table 1). The few GC^+ oligodendrocytes present in the cul-

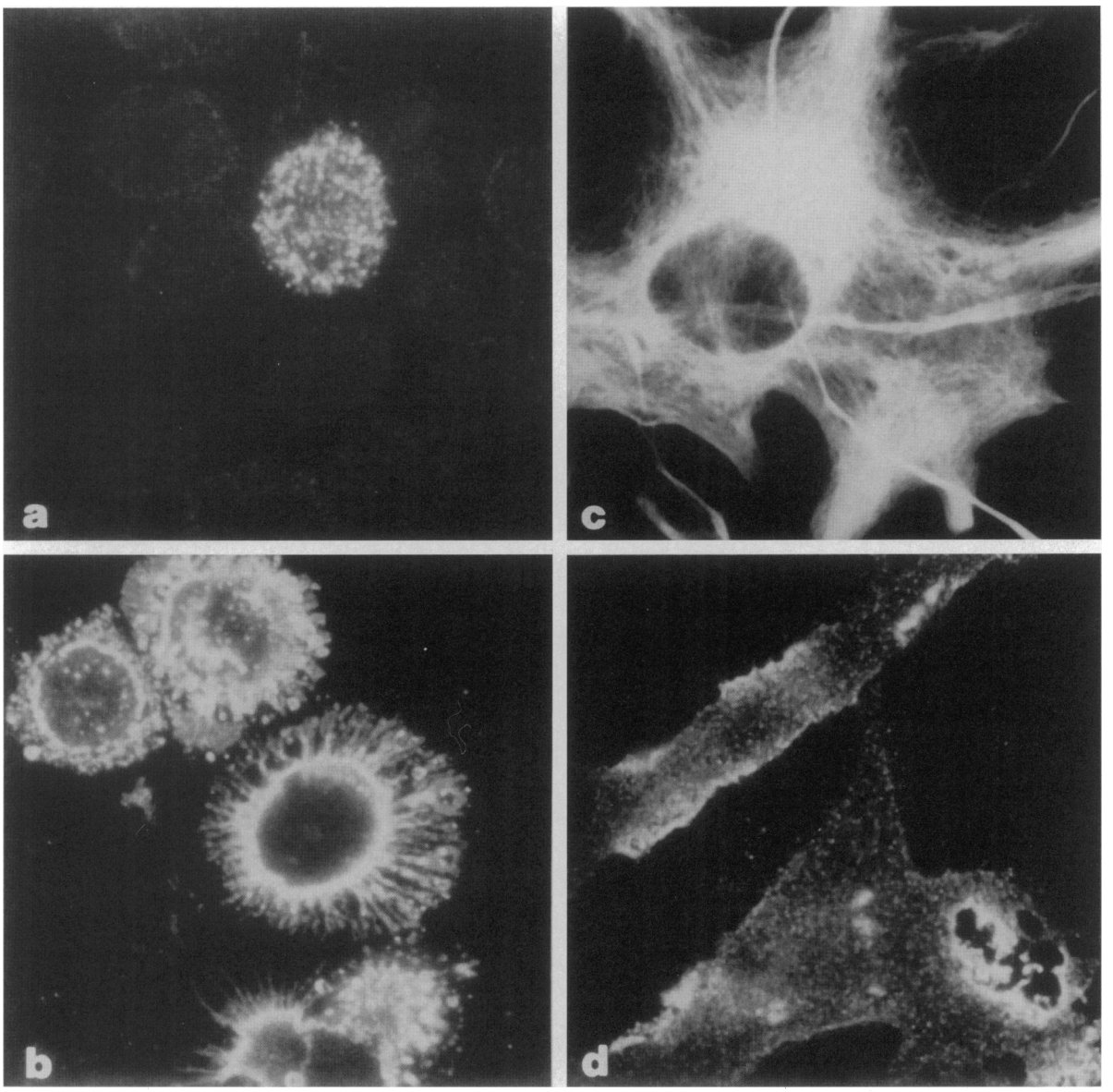
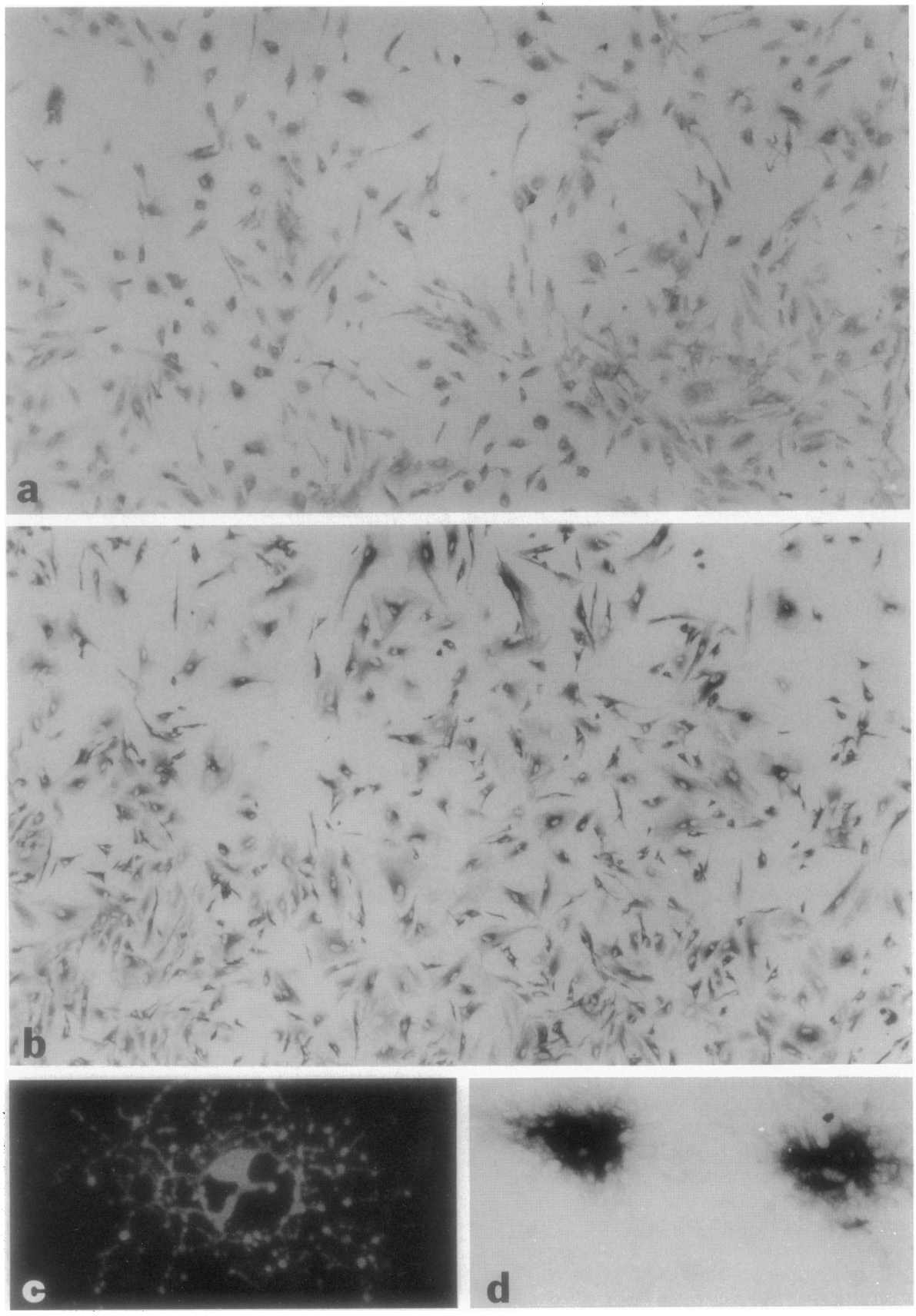


Figure 1. *Indirect immunofluorescence staining. Staining of undifferentiated THP1 cells* (**a**), *PMA-differentiated THP1 cells* (**b**), *and adult astrocytes* (**d**) *for C5aR (CD88) is shown.* **c**: *GFAP staining of adult astrocytes. Cells were cultured on a glass coverslip, ethanol/acetic acid fixed, and immunostained using a mouse monoclonal anti-C5aR (clone W17/1, 0.2 μg/ml) or anti-GFAP (GA5, 1:1000). Cells were then processed as described in Materials and Methods, and fluorescence was visualized on a confocal laser microscope. Magnification, ×400.*

tures did not stain for C5aR (Table 1). No $NF200^+$ neurons were identified in adult brain cultures. Adult brain cultures were also stained using indirect immunoenzymatic techniques, which confirmed the above results (data not shown).

Fetal brain cultures (passage 0) contained astrocytes ($GFAP^+$), neurons (NSE^+, $NF200^+$), fibroblast-like cells ($GFAP^-$, $CD68^-$), and small numbers of microglia-macrophages ($CD11b^+$, $CD68^{++}$) and oligodendrocytes (GC^+). By double immunofluorescence staining we confirmed our previous observations that human fetal astrocytes were stained with all monoclonal and polyclonal anti-C5aRs (Table 1). Neither the $NF200^+$ neurons nor the GC^+ oligodendrocytes were stained with anti-C5aR antibodies (Table 1). Mixed fetal brain cultures at passage 0 contained a few microglia expressing CD68 and CD11b, which, by double staining using the two polyclonal anti-C5aRs, were $C5aR^+$ (Table 1). Primary cultures were enriched for astrocytes according to the protocol described in Materials and Methods and stained for C5aR using an indirect immunoenzymatic (AP) protocol. In Figure 2, astrocytes (culture passage 3) were stained either for C5aR using W17/1 (Figure 2a)


a
b
c
d

or for GFAP using clone GA5 (Figure 2b). All adherent cells in these cultures were $GFAP^+$ astrocytes, and all were $C5aR^+$. Figure 2 also shows an example of fetal oligodendrocyte staining with a monoclonal anti-GC (Figure 2c) and clusters of fetal neurons (NSE^+) overlying a monolayer of astrocytes (NSE^-) (Figure 2). Double immunofluorescence staining confirmed that both fetal oligodendrocytes and neurons were $C5aR^-$, supporting our previous observations that human neuron and oligodendrocyte cell lines do not express C5aR at a detectable level.[16]

When fetal and adult brain cultures were stimulated with various cytokines (200 IU/ml interferon γ, 200 IU/ml interleukin-1β, and 1000 IU/ml tumor necrosis factor α, each for 24 hours) or 50 ng/ml phorbol ester (PMA), no significant difference in the C5aR staining of astrocytes was observed. Cytokine-stimulated neurons and oligodendrocytes did not express a detectable level of C5aR *de novo*.

Light Microscopy and Immunostaining of Tissue Sections

Using the H&E and LFB stains, chronic MS brain contained well-defined areas of demyelination, distributed in a periventricular pattern in the hemispheres. The individual plaques occasionally contained a medium-sized blood vessel that was surrounded by moderate numbers of reactive infiltrating lymphocytes, macrophages, and microglia (all stained for HLA class II; see Figure 3a). At the edge of the plaque there were reactive and hypertrophic astrocytes (large $GFAP^+$ cells) together with microglia (HLA class II^+ and $CD68^+$). In the acute MS cases, the individual plaques were less well defined but contained marked hypercellularity and perivascular accumulations of inflammatory cells and reactive microglia, together with widespread loss of myelin staining.

In HD, the H&E stain showed the caudate nuclei to be significantly atrophied, with evidence of a marked loss of small neurons and reactive astrocytosis.

The cresyl violet stain in PD brains showed marked atrophy of the anterior temporal cortex and a loss of neurons, together with classical Pick inclusion bodies (τ positive) in the remaining neurons of the deeper cortical lamina of the temporal cortex.

In the two HIV encephalitis cases, H&E and LFB stains showed that white matter was diffusely pale, and the gray matter contained characteristic microglial nodules containing numerous reactive microglia (strongly stained for HLA class II and CD68). There were areas of perivascular inflammation, mainly composed of reactive lymphocytes, multinucleated giant cells, and reactive astrocytes.

In the cortical sections from the cases of viral encephalitis, there was a marked perivascular accumulation of reactive lymphocytes and microglia throughout the white matter. A distribution of reactive astrocytes similar to that seen in acute cases of MS was also noted.

For immunostaining, brain tissues from normal controls and various pathological cases (MS, HD, PD, HIV, and encephalitis) were either formalin fixed or snap frozen and immunostained for C5aR using four monoclonal anti-C5aR and the two polyclonal anti-C5aR peptides using the DAB indirect immunostaining protocol. The results of immunostaining are summarized in Tables 2 and 3. In formalin-fixed MS brain, most monoclonal anti-C5aR gave a consistent positive staining of reactive astrocytes and microglia, the best results being obtained with P12/1 (Figure 3d). S5/1 anti-C5aR did not stain formalin-fixed tissue sections (Table 2), but it and all other anti-C5aRs stained reactive astrocytes and microglia in frozen sections of MS brains, indicating that the lack of antibody reactivity was caused by the formalin fixation (Table 2). The endothelia in acute MS brains were also consistently stained for C5aR (Table 2 and Figure 3b). Infiltrating leukocytes were also strongly stained for C5aR, as expected, given that neutrophils and monocytes abundantly express C5aR on their membranes.[1–5] Of the two polyclonal anti-C5aRs, only that raised against the longer peptide 1–30 gave a strong staining of the cellular elements noted above. No difference was seen in the intensity of staining between frozen and fixed tissues sections, making this PAb (at a dilution of 1:1000) the best reagent for C5aR immunostaining of tissue.

To compare the distribution and the level of C5aR expression in normal brain *versus* inflamed brain, all anti-C5aRs were also tested on two samples of normal human brain, both formalin fixed and snap frozen. The results, presented in Table 2, clearly show

Figure 2. *Immunostaining of fetal brain cultures for C5aR. Ethanol/acetic acid-fixed cells were immunostained as described in Materials and Methods.* a: *Cultures enriched for astrocytes (>95%, culture passage 3) and stained for C5aR expression using monoclonal W17/1, AP-conjugated rabbit anti-mouse Ig, and 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium staining. Magnification, ×200.* b: *Cultures as in* a *stained with a monoclonal anti-GFAP (GA5). Magnification, ×200.* c: *Oligodendrocyte in a mixed fetal brain culture (passage 0) stained with a polyclonal anti-GC followed by a fluorescein-conjugated goat anti-rabbit Ig. GC^+ oligodendrocytes were always $C5aR^-$. Magnification, ×1000.* d: *Clusters of NSE^+ neurons in mixed fetal brain cultures (passage 0) incubated with a monoclonal anti-NSE followed by AP-conjugated rabbit anti-mouse Ig. Neuron clusters were found on the monolayer of NSE^- astrocytes. NSE^+ neurons were always $C5aR^-$. Magnification, ×400.*

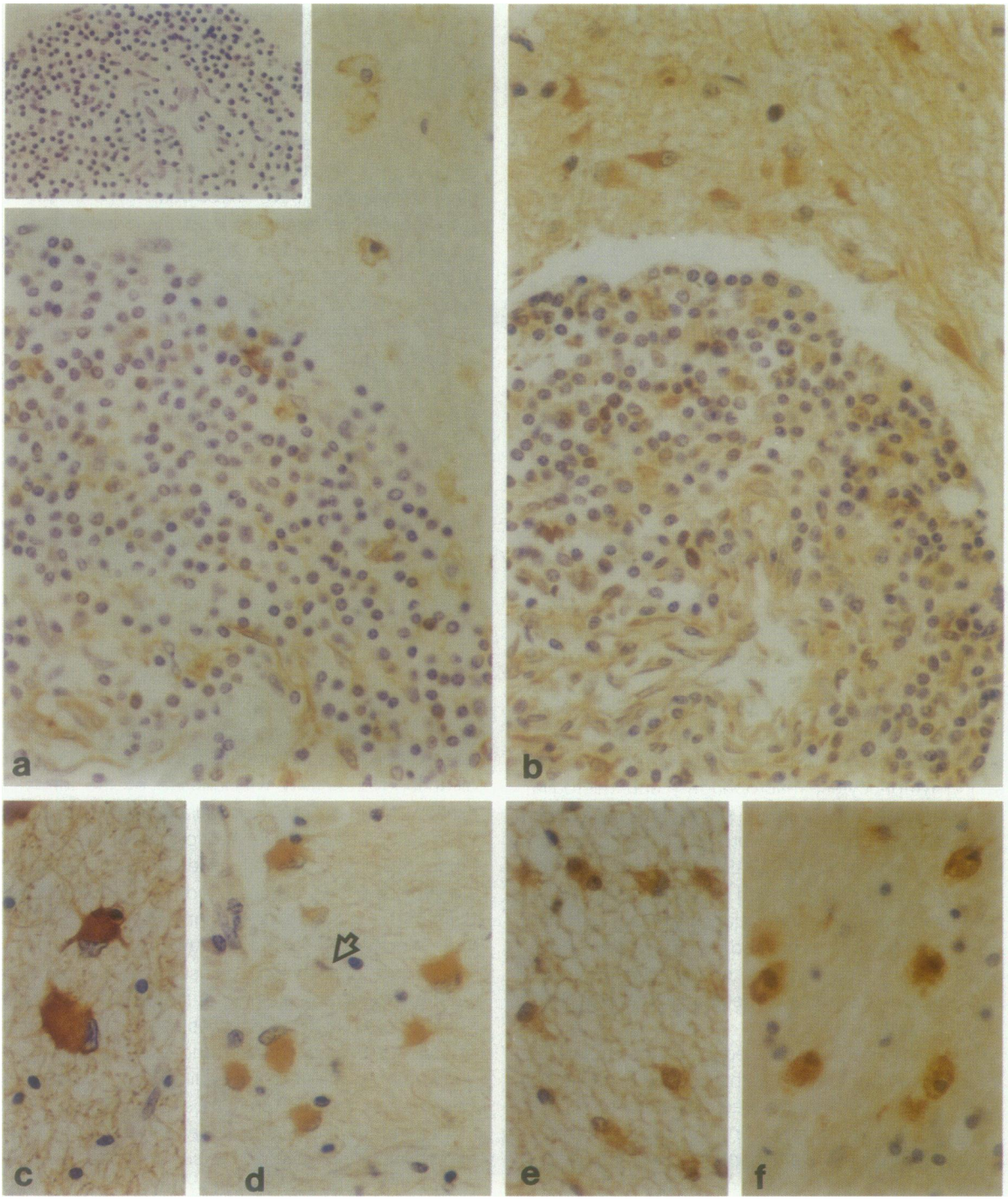


Figure 3. *Immunoperoxidase/DAB staining of rehydrated paraffin wax sections of brain tissue from a case of MS* (a–d) *and from the caudate nucleus of HD* (e *and* f). *The sections were lightly counterstained with H&E.* a *and* b: *Semiserial sections from an acute MS plaque taken from the white matter of the parietal lobe showing a perivascular accumulation of mainly reactive leukocytes.* a: *Immunostaining with the monoclonal anti-HLA class II (clone LN3); note the delicate staining of the endothelium, microglia, and infiltrating macrophages. Magnification, ×280.* b: *Immunostaining with the monoclonal anti-C5aR (P12/1) showing abundant immunostaining localized to many inflammatory cells surrounding the blood vessel wall, the endothelium, and reactive astrocytes. Magnification, ×280.* Inset *in* a: *Negative control using an irrelevant antiserum. Magnification, ×100.* c *and* d: *Same tissue as in* a *and* b *above immunostained with rabbit anti-GFAP, showing intense staining of reactive astrocytes in the white matter* (c, *magnification, ×560) and anti-C5aR p12/1* (d, *magnification, ×560). Note that the astrocytes are more positive than the reactive microglia* (arrow) *in acute MS plaque tissue.* e *and* f: *Formalin-fixed tissue sections from the caudate nucleus in HD samples immunostained for C5aR using anti-C5aR P12/1. Both the astrocytes* (e) *and microglia* (f) *were strongly stained for C5aR in this tissue. Magnification, ×560.*

Table 2. *Immunohistochemistry of Formalin-fixed and Frozen Brain Tissue Sections: Normal and MS Brains*

Antibody	Normal brain, formalin fixed			MS brain, formalin fixed			MS brain, snap frozen		
	Astrocyte	Microglia	Endothelium	Astrocyte	Microglia	Endothelium	Astrocyte	Microglia	Endothelium
PAb anti-GFAP	+++	–	–	+++	–	–	++	–	–
PAb anti-NF200	–	–	–	–	–	–	ND	ND	ND
MAb anti-HLA-Dr	–	+/–	–	–	+++	+	–	+++	+/–
MAb anti-CD68	–	+	–	–	++	+	ND	ND	ND
MAb anti-C5aR (S5/1)	–	–	–	–	–	–	+/–	++	+
MAb anti-C5aR (W17/1)	–	–	–	+	+	+	+	+++	++
MAb anti-C5aR (D12/1)	–	–	–	+/–	+/–	+	+	++	+
MAb anti-C5aR (P12/1)	–	–	–	++	+	+	ND	ND	ND
PAb anti-C5aR peptide 1-30	+/–	+	+/–	+++	+++	+	++	+++	++

that C5aR is expressed at a very low level in normal brain. Only the polyclonal anti-C5aR peptide 1–30 gave a weak but reproducible staining on astrocytes, microglia, and endothelial cells in fixed and unfixed tissue.

The monoclonal anti-C5aR P12/1 was used to analyze expression of C5aR in formalin-fixed tissue from a number of different brain pathologies, and the results are summarized in Table 3. In all these diseases, astrocytes and microglia expressed C5aR at a much higher level than in normal brain. Reactive astrocytes (abundant cytoplasm and high GFAP) expressed more C5aR than nonreactive fibrillar astrocytes (low GFAP). Figure 3 shows an example of HD brain, with astrocytes (Figure 3e) and microglia (Figure 3f) strongly stained for C5aR. Endothelium staining was found in MS, HIV encephalitis, and non-HIV viral encephalitis but not in HD or PD. In each of these diseases, expression of the C5aR by endothelial cells was associated with a massive infiltration of C5aR-positive leukocytes (Figure 3b).

Neurons, axons, oligodendrocytes, and myelin were consistently negative for C5aR with all the available reagents in all brain tissues examined (data not shown).

Discussion

We report here the expression of the C5aR (CD88) by cells derived from the fetal and adult brain and *in situ* in the inflamed brain. Fetal and adult astrocytes and microglia but not neurons or oligodendrocytes expressed C5aR *in vitro*. Inflammatory cytokines neither up-regulated expression on positive cells nor induced expression on negative cells. By immunohistochemistry, using four different well-characterized monoclonal anti-C5aRs and two polyclonal anti-C5aRs, we found that the expression of the C5aR was very low in normal brain but was up-regulated in five different brain diseases: MS, HD, PD, HIV encephalitis, and non-HIV viral encephalitis. C5aR was highly expressed on astrocytes and microglia in all these conditions and on endothelial cells in association with blood-brain barrier damage. These observations demonstrate that C5aR is an excellent marker for inflammation of the brain and suggest that expression of C5aR by glial cells may be a contributory factor in the initiation or perpetuation of brain inflammation and injury.

C5a anaphylatoxin is a powerful chemoattractant released at the inflammatory site during activation of

Table 3. *Expression of C5aR in Other Brain Disorders: Immunostaining Using MAb anti C5aR (clone P12/1) on Formalin-fixed Tissues*

Disorder	Area of pathology	No. of Cases	Astrocyte		Microglia		Perivascular macrophage	Neuron	Endothelium	Oligodendrocyte
			Q	R	Q	R				
MS	Acute plaques	4	+	++	+/–	+	+++	–	+	–
HD	Caudate nucleus	16	++	+++	+++	+++	NA*	–	–	–
Picks	Temporal hippocampus	6	+	++	+++	+++	NA	–	–	–
HIV	Temporal	2	+	++	+++	+++	+++	–	+/–	–
Encephalitis	Cortex	2	+	++	+++	+++	++	–	+	–
Normal	Temporal	5	–	–	–	–	NA	–	–	–

Quiescent (Q) and reactive (R) cells were identified according to their morphology. For example, Q microglia had a fibrillar structure with a small nucleus and a little cytoplasm, whereas the R microglia had a balloon shape, with abundant cytoplasm and a large nucleus. R and hypertrophic astrocytes were strongly stained for GFAP (a typical example can be seen in MS brain tissue section stained for GFAP, Figure 3c).

* NA, not applicable.

the complement system. Diffusion of C5a through the interstitial compartment of a tissue will recruit and stimulate peripheral immunocompetent cells bearing the specific C5aR. Neutrophils and macrophages abundantly express C5aR and respond to a concentration gradient of C5a by leaving the blood stream and migrating toward the inflammatory site, where they play a role in clearing the inflammatory stimulus. C5a also primes the infiltrating cells to release cytokines such as interleukins 1, 6, and 8.[5–11] It has recently been shown that nonmyeloid cells also express a C5aR identical to that described on monocytes. The list now includes hepatocytes, bronchial and alveolar epithelial cells, vascular smooth muscle cells, endothelial cells, and skin mastocytes.[12–15] Hepatocyte expression of C5aR has been implicated as a key player in the induction of the acute-phase response,[13] and endothelial C5aR may play a role in the up-regulation of adhesion molecules in response to inflammation.[15] The first evidence that C5aR might be expressed in the brain came from studies of the effects of injection of C5a directly into rodent brains. It was proposed that C5a acted at a specific receptor to modulate catecholamine activity in rat brains.[23,24] These authors observed a specific and saturable binding of ^{125}I-C5a on rat brain slices, which was blocked by unlabeled, competing C5a but not unlabeled, noncompeting C3a. *In vitro* experiments also showed that rat astrocytes and microglia but not the 0-2A progenitor were able to migrate in response to a gradient of C5a.[25] We and others have recently described the expression of a functional C5aR on cultured human glial cells.[16,26] Our demonstration of C5aR expression *in situ* on astrocytes and microglia in association with inflammation provides crucial evidence for a role of glial cell C5aR expression in brain physiology and pathology.

A role of complement in pathology has previously been suggested in each of the diseases studied.[16,17] In each there is evidence for local activation of the classical pathway, and the cytolytic membrane attack complex has been implicated in demyelination and neurodegeneration.[17,18] C5a is another powerful effector molecule generated during complement activation, and we propose here that release of C5a at the inflammatory site will attract and activate C5aR-positive glia. Whether C5a itself or other factors produced during inflammation cause up-regulation of expression of C5aR on glia remains uncertain. Microglia will be recruited and activated to release proinflammatory cytokines and to phagocytose. Astrocytes will also be recruited and activated, but their role in the perpetuation or resolution of inflammation remains uncertain. C5a may also contribute to changes in blood-brain barrier integrity by binding C5aR expressed on endothelial cells and up-regulating expression of adhesion molecules.

To test these hypotheses *in vivo*, we plan to examine whether a neutralizing anti-C5aR or a C5a competitive antagonist (peptide) can block experimental inflammation in the rat central nervous system. These studies may suggest novel approaches for the therapy of inflammation in human brain diseases.

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