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The Receptor for Complement Anaphylatoxin C3a Is Expressed by Myeloid Cells and Nonmyeloid Cells in Inflamed Human Central Nervous System: Analysis in Multiple Sclerosis and Bacterial Meningitis

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The complement anaphylatoxins C5a and C3a are released at the inflammatory site, where they contribute to the recruitment and activation of leukocytes and the activation of resident cells. The distribution of the receptor for C5a (C5aR) has been well studied; however, the receptor for C3a (C3aR) has only recently been cloned, and its distribution is uncharacterized. Using a specific affinity-purified anti-C3aR peptide Ab and oligonucleotides for reverse transcriptase-PCR analysis, C3aR expression was characterized in vitro on myeloid and nonmyeloid cells and in vivo in the brain. C3aR was expressed by adult astrocytes, astrocyte cell lines, monocyte lines THP1 and U937, neutrophils, and monocytes, but not by K562 or Ramos. C3aR staining was confirmed by flow cytometry, confocal imaging, and electron microscopy analysis. A 65-kDa protein was immunoprecipitated by the anti-C3aR from astrocyte and monocyte cell lysates. Our results at the protein level were confirmed at the mRNA level. Using reverse transcriptase-PCR, Southern blot, and sequencing we found that C3aR mRNA was expressed by fetal astrocytes, astrocyte cell lines, and THP1, but not by K562 or Ramos. The astrocyte C3aR cDNA was identical with the reported C3aR cDNA. C3aR expression was not detected in normal brain sections. However, a strong C3aR staining was evident in areas of inflammation in multiple sclerosis and bacterial meningitis. In meningitis, C3aR was abundantly expressed by reactive astrocytes, microglia, and infiltrating cells (macrophages and neutrophils). In multiple sclerosis, infiltrating lymphocytes did not express C3aR, but a strong staining was detected on smooth muscle cells (pericytes) surrounding blood vessels. The Journal of Immunology, 1998, 160: 3543–3554.

The complement system is an important component of the innate immune system, with the capacity to recognize and eliminate a large variety of pathogens without the recruitment of elements of the adaptive immune system (1, 2). Among the active products conferring this capacity, formation of the membrane attack complex and deposition of opsonins on the membrane of the target cell are key elements for the efficient removal of pathogens. However, the anaphylatoxins released into the fluid phase are critical, in that they attract and activate phagocytic cells and may also activate resident cells at the inflammatory site. The complement anaphylatoxins C5a and C3a are powerful chemottractants that recruit polymorphonuclear cells (PMN) and macrophages into the inflammatory site and activate the cells to phagocyte invading pathogens. C3a and C5a also contribute to the activation of resident cells in the infected tissue, which will then express increased levels of cytokines, chemokines, acute phase proteins (such as complement proteins), and adhesion molecules (3–7). C3a and C5a are regulated physiologically by serum carboxypeptidase N, which inactivates by cleaving a single arginine residue from either molecule; some pathogens possess specific peptidases that inactivate the anaphylatoxins (8).

Cells respond to anaphylatoxins via specific receptors. The receptor for C5a (C5aR) was cloned in 1990 (9), and the generation of specific Abs using synthetic peptides enabled the tissue distribution of C5aR to be examined (10, 11). The C5aR was expressed on both myeloid cells (monocytes/macrophages, neutrophils, and eosinophils) and nonmyeloid cells (hepatocytes, epithelia, endothelia, mast cells, vascular smooth muscle, and glial cells) (3–7, 10–12, 13–16). Much less is known about the cellular distribution of the C3aR. C3a binding experiments and assessment of C3a functional effects indicated that a receptor for C3a was present on monocyte/macrophage cells and cell lines, platelets, polymorphonuclear leukocytes, mast cells, and adipocytes (3, 12, 17–23). The human C3aR cDNA has recently been cloned from the HL60 cell line (24), LPS-activated neutrophils (25), and the PMA-differentiated U937 monocyte cell line (26). Northern blot analysis revealed that the 2.2-kb C3aR mRNA was expressed in all tissues, including CNS, central nervous system; MAP, multiple array peptide; GST, glutathione-S-transferase; GFAP, glial fibrillary acidic protein; MNC, mononuclear cells; PE, phycoerythrin; DAB, diaminobenzidine; IF, immunofluorescence; DAF, decay-accelerating factor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

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3 Abbreviations used in this paper: PMN, polymorphonuclear cells; C5aR, receptor for C5a; C3aR, receptor for C3a; MS, multiple sclerosis; BM, bacterial meningitis; CNS, central nervous system; MAP, multiple array peptide; GST, glutathione-S-transferase; GFAP, glial fibrillary acidic protein; MNC, mononuclear cells; PE, phycoerythrin; DAB, diaminobenzidine; IF, immunofluorescence; DAF, decay-accelerating factor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

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particularly in lung, placenta, heart, spleen, and brain (25). However, little is known about the expression of C3aR at the protein level and on nonmyeloid cells in these tissues.

We and others are interested in the role of complement in the brain, and we have shown that a full complement system can be expressed by resident cells in the brain, particularly after cytokine stimulation (for review, see Ref. 27). The brain is an immunoprivileged organ isolated from the peripheral immune system, and we have proposed that complement expressed locally in response to infection or inflammation plays an important role as an anti-pathogen (27). Human and mouse glial cells respond to and are activated by anaphylatoxins (14, 28, 29), and we have recently shown that the C5aR was expressed by astrocytes, the most abundant glial cell type (14). Immunohistochemistry demonstrated that expression of C5aR was low in normal brain but abundant in inflamed brain, suggesting that the anaphylatoxin receptor was involved in the inflammatory process (16).

We here describe the expression of the C3aR by myeloid cells and brain cells in vitro and in brain tissue. C3aR expression was demonstrated in cells and tissue at the protein and mRNA levels, and immunohistochemistry was conducted on normal, multiple sclerosis (MS), and bacterial meningitis (BM) brain tissue sections. In myeloid and glial cells the expressed C3aR was a protein of 65 kDa; expression of the receptor was highly elevated on myeloid cells, astrocytes, microglia, and smooth muscle cells (pericytes) in inflamed CNS tissue. The results further emphasize the important role played by complement activation in CNS inflammation.

Materials and Methods

Peptide synthesis, production of Abs, and affinity purification

A multiple array, 30-aminocid, C3aR peptide PSGFPIEDHETSPLDNSSKLFPSSKLKLFPS (MAP-C3aR peptide) corresponding to amino acid residues 270 to 300 of the C3aR loop (see Fig. 1) was synthesized on an Applied Biosystems Synergy Synthesizer (Applied Biosystems, Piscataway, NJ). Specific IgG antisera were raised in New Zealand White rabbits by repeated s.c. immunization (total of five) with peptide (100 μg/animal/immunization) in CFA using standard protocols. The animals were test-bled at intervals, and the titer of the anti-peptide response was measured. When the anti-peptide response had plateaued, the animals were sacrificed by exsanguination, and the antiserum was stored at −20°C.

Total IgG was purified on glass-bound, protein A-Sepharose (Prosep A, Bioprocessing, Princeton, NJ). Specific IgG was then affinity purified from the total IgG using the same C3aR peptide coupled to Sepharose. The C3aR peptide Sepharose was generated by coupling directly 5 mg of MAP-C3aR peptide with 1.5 g (5–7 ml) of cyanogen bromide-activated Sepharose 4B according to the manufacturer’s protocol (Pharmacia, U.K.). IgG (200–250 mg) was incubated overnight (4°C with mixing) with the MAP-C3aR peptide coupled to Sepharose. After washing repeatedly with PBS, the Sepharose was poured into a column, and Ab was eluted using 0.1 M glycine-HCl buffer at pH 2.5. The yield of affinity-purified anti-peptide IgG was 20 to 25 mg from 200 to 220 mg of total IgG (corresponding to 20–25 mg of rabbit serum).

For FACS analysis, affinity-purified anti-C3aR (1.5 mg) was also biotinylated with 1 mg of biotinamidocaproate-N-hydroxysuccimide (Sigma, Poole, U.K.) and dialyzed to eliminate free biotin. All preparations of Abs were stored at 4°C in PBS/0.1% NaN₃. The specificity and titer of the anti-peptide Ab were assessed in an ELISA using 96-well plates coated with the MAP-C3aR peptide or nonspecific peptides (C3a peptide, WWGKKYRASKLGLAR; MAP-C5aR peptide, MNSSFEINYDHYGT MELISPADGHIHPKKQ) produced in our laboratory (14). In some experiments, the anti-C3aR was blocked by preincubation with various concentrations of the MAP-C3aR peptide before application to the 96-well plate. Specificity was also assessed by flow cytometry on C3aR⁺ (THP1) and C3aR⁻ (Ramos) cell lines and by Western blot analysis of cell lysates and a C3aR loop-glutathione-S-transferase (GST) fusion protein.

To detect C3aR extracted from cells, lysates were immunoprecipitated using anti-C3aR Sepharose. For this purpose, 5 μg of affinity-purified anti-C3aR was coupled to 1.5 g (5–7 ml) of cyanogen bromide-activated Sepharose. For FACS analysis, affinity-purified rabbit "B" anti-C3aR (1.5 mg) was also biotinylated with 1 mg of biotinamidocaproate-N-hydroxysuccimide (Sigma, Poole, U.K.) and dialyzed to eliminate free biotin.

Cells, cytokines, and Abs

Recombinant human IFN-γ, IL-1β, and TNF-α were gifts from Hoffmann-La Roche (Nutley, NJ). PMA, protein A-Sepharose, pepstatin, and leupeptin were obtained from Sigma. Mouse and rabbit Abs against the N-terminal extracellular sequence of the human C5aR have been characterized previously (11, 14). Abs against CD Ags (CD4, CD14, CD16, CD18, CD19, CD35, and CD68) were purchased from Dako (High Wycombe, U.K.). mAbs against CD44 (clones BRIC222 and BRIC235) and CD59 (BRIC229) were obtained from IBGRL (Elstree, U.K.). Other Abs used for immunocytochemistry of adherent cells on coverslips and for immunohistochemistry of tissue sections were mouse anti-glial fibrillary acidic protein (anti-GFAP; clone GA5; 1/1000; Sigma), mouse anti-galactocerebroside (1/1000) (16), mouse anti-neuron-specific enolase (clone BBS/NC/VI-H14; 1/2000; Dako), mouse anti-CD11b (clone 2LPM19c; 1/50; Dako), mouse anti-CD68 (1/50; clones KP1, PG-M1, and EB-M1; Dako), and mouse anti-HLA class II (1/50; clone LN3; Biotest, Sohlhull, U.K.).

Cell preparation and culture

Primary cultures of human fetal astrocytes were grown in our laboratory from tissue supplied by the Medical Research Council Fetal Tissue Bank (London, U.K.). All cultures were established from fetal brain (6- to 10-wk-old fetus) as described previously (14). Primary cultures (passage 0, days 1–7) contained clusters of neurons (demonstrated by immunostaining with an anti-70K neurofilament mAb) that survived 7 to 10 days, lying on a monocellular layer. Some 60 to 80% of the cells in this monocellular layer expressed the astrocytic marker, GFAP; 5 to 7% were microglia, and 5% were endothelial cells.
15 to 40% were fibroblasts. Microglia were removed by shaking (350 rpm, 37°C, for 1.5 h followed by a second shaking at 250 rpm and 37°C overnight). Adherent cells after these two sequential shakings were subcultured in DMEM containing 10% FCS (Life Technologies, Paisley, U.K.), 1% L-glutamine (Life Technologies), and 1% penicillin/streptomycin (Life Technologies). Experiments were conducted from passages 2 to 5, when cultures contained >95% GFAP +ve cells and <5% neurons, fibroblasts, and microglia.

Normal adult temporal lobe tissue was obtained fresh from biopsies of patients undergoing therapeutic resection for intractable epilepsy. The tissue was used for culture of adult astrocytes as previously described (16, 30). The use of human tissue was in accordance with procedures and regulations established by the local ethical committee of the University of Wales College of Medicine.

The two human astrocyte cell lines, T98G and CB193, were cultured and characterized as described previously (14). Neutrophils (PMN) and mononuclear cells (MNC) were isolated from heparinized blood obtained by venepuncture of healthy volunteers. Briefly, leukocytes were separated from erythrocytes by dextran sedimentation using 0.6% (w/v) dextran (Fisons, Loughborough, U.K.). The leukocyte-rich upper layer was then fractionated by layering on Histopaque (Sigma) followed by centrifugation at 220 × g for 25 min at room temperature. MNC were collected from the interface and washed in PBS/BSA. Residual erythrocytes in the PMN-rich cell pellet were removed by hypotonic lysis, and PMN were washed in PBS/BSA.

Cytocentrifuge preparations of cells were stained with Wright’s stain, and >99% PMN were PMN. FACS analysis of PMN preparations revealed that 90 to 95% of cells were CD11b+CD18+, CD16+, CD14+, CD19+, and CD35+. Lymphocytes and monocytes constituted >90% of the MNC preparation, and no contaminating PMN were identified. In the MNC preparation, 10 to 15% of cells analyzed by FACS were strongly CD3+.

Human monocyte-derived cell lines, THP1 and U937, and neuroblastoma cell lines, IMR32 and SKNSH, were obtained from American Type Culture Collection (Rockville, MD). The human endothelial cell line ECV 304, the human erythroleukemia cell line K562, the human B lymphocyte cell line Raji (Raji), and the human glioblastoma cell lines, IMR32 and SKNSH, were obtained from the local ethical committee of the University of Wales College of Medicine.

For CD44 and C3aR staining, adult astrocytes, fetal astrocytes, and astrocyte cell lines were cultured on sterile glass coverslips for 5 to 8 days, and after washing with PBS, cells were fixed with 1% formaldehyde for 20 min. Cells were then washed intensively in PBS/0.2 M glycine to block aldehyde groups. For GFAP immunostaining, cells were fixed and permeabilized with a mixture of 95% ethanol/5% acetic acid for 5 min at −20°C. Abs were used at optimal dilution (1 μg/ml in PBS/1% BSA) and incubated with fixed cells overnight at 4°C in a humid chamber. Affinity-purified rabbit anti-C3aR was tested over the concentration range 7.5 to 0.81 μg/ml in the presence or the absence of competing peptide. After overnight incubations were fixed for 30 min at 37°C with either FITC-labeled secondary Ab (F(ab’2), rabbit anti-mouse IgG (1/100; Dako) or goat anti-rabbit IgG (Sera-Lab, Sussex, U.K.) or peroxidase-labeled secondary Ab (rabbit anti-mouse IgG (1/100; Bio-Rad, Richmond, CA) or goat anti-rabbit IgG (1/100; Bio-Rad)). After intensive washing, coverslips for fluorescence microscopy were mounted in Citifluor (Citifluor, London, U.K.) and sealed. For peroxidase immunostaining, coverslips were incubated in a solution of DAB/H2O2 (Sigma) before hematoxylin counterstaining and mounting. Fluorescence was imaged by confocal laser scanning microscopy on a Leica TCS microscope (Leica, Heidelberg, Germany). Twelve optical sections were collected per field at 0.3- to 0.5-μm intervals from the bottom to the top of the cell. Sections were then assembled as extended focus views, or individual sections were viewed as a gallery. DAB immunostaining (brown positive staining) was photographed on a Leica DMLB microscope with brightfield at two magnifications (×500 and ×1250).

Double staining of the CB193 astrocyte cell line for CD44 and C3aR was performed in an identical manner, except that both Abs were applied to the same coverslip and were detected using a rhodamine-conjugated goat anti-mouse IgG (Sigma) and a FITC-conjugated goat anti-rabbit IgG (Seraphab), respectively. Fluorescence was imaged by confocal microscopy using specific cut-off filters for FITC and rhodamine.

Sources of tissues, processing, and immunohistochemistry

Brain tissue was obtained locally at autopsy or from specialist tissue collections. Tissue was collected from individuals with a variety of brain disorders (demyelination and CNS infection) and from normal surgical controls. Tissue from cases of MS (three acute plaques and one chronic plaques) were obtained from Dr. Jia Newcombe (MS Society Laboratory, London, U.K.) and locally (J. W. Neal, Neuropathology Laboratory, Cardiff, U.K.). Tissues from four cases of BM were obtained locally, all of which were characterized by a marked infiltration of neutrophils in the meninges. Normal control brain tissues was obtained at autopsy or brain surgery from individuals with no evidence of neurodegenerative disease, ischemia, or gliosis. Autopsy samples were obtained at a similar postmortem interval (maximum of 30 h) as the disease samples. Brains were cut coronally, and individual blocks from areas of the brain 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.

Rehydrated parafin sections were counterstained with hematoxylin/eosin to display morphology, and Luxol fast blue stain was used to identify demyelinating plaque areas in MS tissue (16). Rehydrated paraffin wax sections and cryosections (8 μm) from normal and diseased brains were immunostained with different dilutions of the affinity-purified anti-C3aR (7.5 to 0.81 μg/ml) and anti-cell markers diluted in PBS/BSA using an indirect immuno-horseradish peroxidase/DAF method as described previously (32). Peroxidase-labeled swine anti-rabbit IgG and rabbit anti-mouse IgG (Dako; 1/100 dilution) were used as secondary Abs.

For double immunofluorescence (IF), tissue sections were incubated simultaneously with the rabbit anti-C3aR and mouse anti-brain cell markers (clone G5A for GFAP and clone LN3 for HLA class II) followed by FITC-labeled goat anti-rabbit (Sera-Lab) and rhodamine-labeled goat antimercury (Sigma) Abs. Fluorescence was imaged on a Leica DMLB epi-fluorescence microscope using specific filters.

Cell lysates, Western blotting, and immunoprecipitation

Western blotting was performed on cell cultures (astrocytes and cell lines) solubilized in PBS containing 2% Nonidet P-40 together with enzyme inhibitors as previously described (14). To enrich for C3aR before Western blotting, cell lysates were immunoprecipitated. Freshly made cell lysate (1 ml; 2 × 10^7 cells/ml) was incubated with mixing overnight at 4°C with 50 μl of affinity-purified rabbit anti-C3aR Sepharose. After five washings with 0.5% Nonidet P-40 in PBS, C3aR retained on the Ab-Sepharose was eluted.
by mixing in an Eppendorf microfuge with 50 μl of 0.1 M glycine, pH 2.5, for 30 min at room temperature. Sepharose was removed by centrifugation at 10,000 × g for 10 min, and the eluted protein was diluted in an equal volume of SDS-PAGE sample buffer. The incorporation of the glycine elution step prevented the release of rabbit anti-C3αR, simplifying interpretation of the Western blots. THP1 and Ramos cell lysates were tested as positive and negative controls for C3αR expression to validate the immunoprecipitation protocol. Samples were run at 150 μg/lane (754 bp) or rabbit anti-decay-accelerating factor (anti-DAF; CD55) also produced in our laboratory (0.25 μg/ml), essentially as previously described (14). After washing and incubation with peroxidase-labeled goat anti-rabbit IgG (Bio-Rad; 1:4000), the blots were developed using the enhanced chemiluminescence system (ECL, Pierce, Chester, UK). Prestained broad range protein markers from New England Biolabs (Beverly, MA) were used as m.w. standards. Human THP1, unstimulated or differentiated with PMA (10 nm/10 ml, 3 days), were used as the C3αR-positive control.

To eliminate the possibility of cross-reactivity of affinity-purified anti-C3αR Ab with C5αR, PMA-differentiated THP1 cell lysate (1 ml; 2 × 10⁶ cells) was immunoprecipitated with W17 mouse monoclonal anti-C5αR preadsorbed on protein A-Sepharose (Sigma; 20 μg of Ab for 6.25 mg of PAS). After elution in Laemmli buffer, samples were tested by Western blot using the affinity-purified anti-C3αR.

RNA extraction and RT-PCR for C3αR mRNA
Total RNA from unstimulated and stimulated cultures of human astrocytes and cell lines was prepared using the Ultraspec RNA isolation system (Biotech, Houston, TX) according to the manufacturer’s instructions. RNA integrity was confirmed on agarose gels, and concentrations were determined from absorbance at 260 nm. Before reverse transcription, total RNA (50 μg) was treated for 20 min at 37°C with 10 μl of RQ1 RNase-free DNase (Promega, Madison, WI) in 100 μl of buffer (40 mM Tris/HCl (pH 8), 10 mM NaCl, 6 mM MgCl₂, and 10 mM CaCl₂) and 200 U of RNAsin (Promega) to remove all trace of DNA. The mixture was then phenol and chloroform extracted. The aqueous phase was ethanol precipitated, and the pellet after centrifugation was resuspended in diethylpyrocarbonate-treated water.

The reverse transcription was conducted at 37°C for 120 min in 30 μl (final volume) with 2 μg of total RNA (DNA-free), 60 μl of RNAsin (Promega), 1 mM dNTPs (Bioline, London, U.K.), 250 pmol of random hexamer primers (pdN6 from Pharmacia), and 400 U of Moloney murine leukaemia virus-RT (Life Technologies, Paisley, U.K.) in the reaction buffer (10 mM Tris/HCl, 15 mM KCl, 0.6 mM MgCl₂, and 5 mM DTT). The absence of contaminants was routinely checked by RT-PCR assays of negative controls. The RT-PCR product was purified by the PCR DNA purification system (Biotecx, Houston, TX) according to the manufacturer’s instructions. RNA extraction and RT-PCR for C3αR mRNA

PCR was conducted with 3 μl of reverse transcribed RNA mixture in a 50-μl final reaction volume with 100 pmol of each C3αR primer (generated in-house on a Beckman oligo synthesizer, Beckman, Palo Alto, CA) in 10× buffer (Promega) containing 1.5 mM MgCl₂, 200 μM dNTP, and 1.25 U of Taq DNA polymerase (Promega). The PCR protocol used was: denaturation step at 94°C for 4 min; five cycles of 94°C for 30 s, annealing 60°C for 1 min, and extension at 72°C for 2 min; 25 cycles of 94°C for 10 s, 60°C for 30 s, and 72°C for 45 s; and elongation at 72°C for 15 min. PCR was performed in a Hybaid (Teddington, U.K.) Omnimag thermocycler.

Seven oligonucleotides were selected from the published C3αR cDNA sequence (23–25). Their sequences from 5’ to 3’ and their positions in parentheses are given according to the sequence published by Roglic et al. (EMBL accession no. M62505). The C3αR primers were: act-1, CTA CAA TGA GCG TGG TGG (sense, 511); and act-2, CTT CAT GGC ATT GGT GGA GA (antisense, 508). The sequences of C3αR (EMBL M33197) primers were: GAPDH-UP, CAA GAA TGT CTT CAT CA; and GAPDH-DN, TGA CTC TGC CCA CAG CCT TG.

RT-PCR analysis for complement receptor type 2 (CR2), C5αR, and C4 mRNAs was also conducted on all RNA samples to confirm the specificity of the RT-PCR protocol (20, 33). The sequences of human CR2 primers (EMBL J03564) were: CR2 ATG GGT GGT ACC CTG (181) and ACA CAG GGT GGT AGT CGT (503). The sequences of human C5αR primers (EMBL no. M62505) were: GAC CAG AAC ATG AAC TCA CC T (16) and TGG CGC CTA CAT TGC CTG (1083). The sequences of human C4 primers (EMBL no. K12403) were: CGG GTC TTT GCC CTG CAT GA (515) and CTT CAC CTC RAA GTT GGG AA (773). Samples of RT-PCR products were loaded onto a 1% agarose gel in 1× TBE buffer, separated by electrophoresis at 50 to 100 mA, and transferred onto nylon membranes (Hybond N⁺, Amersham, Aylesbury, U.K.) for Southern blotting. Ladders (123 bp and 1 kb; Life Technologies) were used as DNA size markers. All C3αR cDNAs obtained after RT-PCR of the astrocyte cell line CB193 were purified (Quiagene PCR purification column, Qiagen, Hilden, Germany). The PCR products were loaded onto a 1% agarose gel in 1× TBE buffer, separated by electrophoresis at 50 to 100 mA, and transferred onto nylon membranes (Hybond N⁺, Amersham, U.K.).

cDNA probes, nucleotide sequencing, and Southern blot
The human C3αR cDNA probe was obtained from PMA-differentiated U937 RNA by RT-PCR using two C3αR oligonucleotides (754 and 1391). The RT-PCR product was purified by the PCR DNA purification system (Promega) and subcloned in the PGEM-T plasmid (Promega, Southampton, U.K.). Positive bacterial clones were identified by blue/white screening and were selected. Inserts were PCR amplified using primers SP6 and T7 (devised in-house) derived from vector sequence flanking the insert site and then sequenced as described above. The probe isolated by this procedure was 100% homologous with the reported C3αR cDNA (24) between positions 754 and 1391 bp.

For Southern blotting the C3αR probe was labeled by the random oligo procedure with [α-32P]-dCTP (3000 Ci/mmol; Redivue, Amersham). Blots were prehybridized (2 h) and hybridized (16–20 h) in a hybridization oven at high stringency (50% deionized formamide, 5× SSPE, 1% SDS, 5× Denhardt’s reagent, 5% dextran sulfate, and 100 μg/ml denatured and fragmented herring sperm DNA). Posthybridization washings were performed in 0.1×SSPE/0.1% SDS, two times at room temperature for 1 h, 0.1×SSPE/0.1% SDS for 1 h at 68°C, and 0.1×SSPE/0.1% SDS for 1 h at 68°C. Blots were then exposed to Kodak film (Eastman Kodak, Rochester, NY) for 5 to 10 min at room temperature.

GST-C3αR loop fusion protein
To further confirm the specificity of the anti-C3αR peptide we generated a fusion protein by cloning the cDNA encoding the second C3αR extracellular loop (between TM4 and TM5 domains, amino acids 161–332) in a GST fusion protein system (Pharmacia). The C3αR loop cDNA was produced by RT-PCR from PMA-differentiated THP1 RNA using two specific oligonucleotides (F-C3αR-s (562, CGG GAT CCC GGG AAA TTT CCA CTA CAG AC) and R-C3αR-s (1077, CGG GAT CC TCA GGT GGG TGC ACT TGA TGC TTG) and Vent DNA polymerase (New England Biolabs, Hertfordshire, U.K.) to minimize PCR errors. The sequence TCA (double underlined) was introduced in the downstream primer to create a stop codon TGA. A BamHI site (underlined) was introduced in both primers and was used to clone the C3αR cDNA into the pGex-2T expression vector. After transfection in Escherichia coli (strain BL21, Pharmacia), plasmid from individual bacterial colonies was tested to confirm the C3αR insert orientation and the fidelity of the C3αR cDNA sequence. The expression of the fusion protein was induced by the addition of 1 mM isopropyl-β-d-thiogalactopyranoside (Promega) in 0.5 I bacterial culture (OD₅₆₂₅ mm = 0.8) for 2 to 3 h. Bacteria were pelleted at 5000 × g for 30 min, and lysates were prepared using 1% Nonidet P-40PBS. Crude lysate was analyzed by Western blot, and the expressed fusion protein was affinity purified on glutathione-Sepharose 4B according to the manufacturer’s instructions. The predicted size of the fusion protein was 50 kDa (30 kDa for GST and 19.4 kDa for the C3αR loop).

Results
Production of a specific anti-C3αR Ab
A 30 amino acid sequence from the second extracellular loop (amino acids 270–300; inset in Fig. 1) was chosen for Ab production based upon predictions of hydrophilicity/antigenicity. This peptide had no significant homology with any other cloned protein reported in the BLAST database and had no homology with the human C5αR. The C3αR peptide was synthesized as a multiple array on a lysine core and was used for immunization of rabbits.
The antiseraum was purified by affinity chromatography on peptide-Sepharose. The yield of affinity-purified anti-C3aR Abs was approximately 10% of the applied IgG. Affinity-purified anti-C3aR IgG was used throughout the study. The specificity of the anti-C3aR was tested first by staining the THP1 monocyte line and the Ramos B cell line followed by FACS analysis (Fig. 1). THP1 was stained with peroxidase-conjugated secondary Ab, and specific binding of the Ab was assessed by developing the plate with OPD substrate (Dako) and measurement of the OD at 490 nm. The y-axis represents the mean of OD readings of three wells for each dilution of Ab. The three curves are the results of three independent experiments.

Expression of C3aR by neutrophils, monocytes, PMA-differentiated THP1, and other cells

We have previously described the expression of C5aR by leukocytes (primary cells and cell lines); the same approach was used to characterize the expression of C3aR using FACS analysis. THP1, undifferentiated or differentiated with PMA (3 days), were stained for C3aR; PMA-differentiated cells had a mean fluorescence double that of undifferentiated cells (Table I). We have previously shown that PMA differentiation increased the expression of C5aR on THP1 by a factor of 10- to 20-fold, and these results were here confirmed in parallel with the measurements of C3aR expression (Table I). To characterize in more detail the regulation of C3aR expression by monocyte cell lines, U937 were cultured for 48 h in the presence of three different concentrations of recombinant cytokines (IFN-γ, IL-1β, and TNF-α) or phorbol ester (PMA). C3aR expression on the cell membrane was analyzed by FACS, and the results are presented in Table II. PMA increased the expression of C3aR, but only by a factor of 1.5 at 48 h poststimulation; the most dramatic effect was observed after stimulation of U937 with IFN-γ. Even at a very low concentration (10 IU/ml) IFN-γ increased C3aR expression by 2-fold, and at 1000 IU/ml, C3aR expression was elevated by 6-fold. TNF-α and IL-1β had no effect on C3aR expression by U937 (Table II). IFN-γ also up-regulated C3aR expression by THP1. Neutrophils and monocytes both expressed C3aR, whereas the various lymphocyte subsets were all negative (Table I). C3aR was not detected on the membranes of the undifferentiated Raji 3 B cell line, the K562 erythroleukemia cell line, the Molt 4 T lymphocyte cell line, or the YT NK cell line (data not presented).

Astrocyte cell lines and fetal and adult astrocytes express constitutively membrane C3aR

Two astrocyte cell lines were tested by FACS for C3aR expression. The well-differentiated astrocyoma cell line CB193 expressed twice as much C3aR as the undifferentiated glioblastoma cell line T98G (Table I). Both lines express the astrocyte-specific marker GFAP and have been extensively used as a model of the human astrocyte (33). Indirect IF and confocal microscopy confirmed that C3aR was expressed abundantly on the membrane of the CB193 astrocyte cell line (Fig. 3, b and c). No staining was detected in the nucleus or the cell cytoplasm. C3aR staining was patchy, a pattern identical with that described for expression of C5aR on astrocyte lines (14). The C3aR staining intensity and pattern were reproducible (n > 10), specific, and blocked by the competitive peptide. To exclude the possibility that the patchy C3aR staining was an artifact of the immunocytochemistry procedure, we simultaneously stained CB193 for membrane-associated CD44 and C3aR. Figure 3, d and e, clearly shows that CD44 membrane staining was distributed homogeneously, whereas C3aR staining on the same cells was patchy and clustered.

Expression of C3aR was also examined on primary astrocytes cultured from fetal (n = 5) and adult (n = 2) brain using a classic immunoperoxidase/DAB staining technique. Fetal astrocytes were faintly but consistently stained (data not shown), whereas adult astrocytes were strongly stained by anti-C3aR (Fig. 4). Adult astrocytes are adherent cells with long, ramified processes and stain...
strongly for GFAP (Fig. 4a). The C3aR staining on adult astrocytes was again patchy and confirmed our results with the CB193 astrocyte cell line (Fig. 4d and Fig. 3, b, c, and e). Neurons and oligodendrocytes in fetal brain cultures did not express C3aR at a detectable level (not shown), whereas microglia in fetal and adult brain cultures were stained for C3aR (Fig. 4b). Adult and fetal brain cultures and CB193 were stimulated for 24 h with various cytokines (IFN-γ, IL-1β, and TNF-α) or with PMA. No change in C3aR expression was detected on any cell type in the cultures (data not shown).

C3aR on monocyte lines and astrocytes is a 65-kDa protein

Although the GST-C3aR fusion protein was readily detected by immunoblotting (Fig. 5A) and DAF was easily detected in cell lysates (Fig. 5B), no positive results were obtained for C3aR from cell lysates. To improve sensitivity, cell lysates were first immunoprecipitated on Sepharose-bound anti-C3aR. Western blotting of immunoprecipitates revealed a protein with an apparent molecular mass of 65 kDa in lysates from unstimulated CB193, undifferentiated THP1, and PMA-differentiated THP1 (Fig. 5C). The intensity of the 65-kDa band was greater in the PMA-stimulated THP1 lane compared with that of undifferentiated THP1. No protein was detected when Ramos or K562 cell lysates were immunoprecipitated under the same conditions. When THP1/PMA cell lysate was immunoprecipitated with either Sepharose-bound polyclonal anti-C5aR or monoclonal anti-C5aR (W17/1) adsorbed on protein A-Sepharose to enrich the preparation for C5aR, no protein was detected upon Western blotting with anti-C3aR (not shown).

Table II. Effects of IFN-γ, IL-1β, TNF-α, and PMA on C3aR expression by monocyte cell line U937

<table>
<thead>
<tr>
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<th>FACS Analysis (mean of fluorescence)</th>
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<tbody>
<tr>
<td></td>
<td>IFN-γ (48 h, IU/ml)</td>
</tr>
<tr>
<td></td>
<td>10</td>
</tr>
<tr>
<td>CD44 (B222)</td>
<td>638.8</td>
</tr>
<tr>
<td>CR3 (OKM1)</td>
<td>59.4</td>
</tr>
<tr>
<td>C5aR (P12/1)</td>
<td>105.9</td>
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<tr>
<td>C3aR(α-pept)</td>
<td>75.77</td>
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* U937 monocyte cell line (10⁵ cells/ml) was cultured for 48 h in a 24-well plate, in medium alone (Non Stim.) or with medium supplemented with three different concentrations of IFN-γ, IL-1β, TNF-α, or phorbol ester (PMA). Cells were then analyzed by FACS for CD44 (mouse clone BRIC 222), CR3 (mouse clone OKM1), and C5aR (mouse clone P12/1). A significant difference of 50% in the level of expression between control cells and stimulated cells is presented in bold. Data from one typical experiment and the results were confirmed in two additional separate experiments.

* Biotin-conjugated rabbit anti-C3aR (3.75 µg/ml) was used in this experiment with RPE-conjugated streptavidin (1/200, Jackson ImmunoResearch).

FIGURE 3. Immunolocalization of C3aR on the human astrocyte cell line CB193 by indirect IF and confocal microscopy analysis. IF was conducted on CB193 astrocytes cultured and fixed on glass coverslips and incubated with Abs against two membrane receptors, CD44 and C3aR. Cells were incubated with either a) mouse anti-CD44 clone BRIC222 (TCS; 1/200) followed by an FITC-conjugated rabbit anti-mouse Ig Ab, or b) and c) affinity-purified rabbit anti-C3aR (3.75 µg/ml) followed by an FITC-conjugated goat anti-rabbit Ig Ab. In d and e, cells were double immunostained for CD44 and C3aR. Cells were simultaneously incubated with BRIC222 and anti-C3aR, followed by rhodamine-conjugated goat anti-mouse Ig (Sigma) and FITC-conjugated donkey anti-rabbit Ig (SeraLab). IF was analyzed by confocal microscopy. Extended focus views demonstrated that fluorescence was localized on the membrane for both Abs. CD44 staining was homogeneously distributed on the membrane (a and d); C3aR staining (b, c, and e), however, was patchy and localized to restricted areas of the cell membrane. Optical sections of the image did not show any CD44 or C3aR staining in the nucleus or the cytoplasm of CB193 (data not shown). The arrowheads mark the locations of cells in the same field either on the rhodamine channel (CD44 staining; d) or on the FITC channel (C3aR staining; e). Magnifications: a, c, d, and e, ×500; b, ×1250.
C3aR mRNA expressed by astrocytes is 100% identical with the leukocyte C3aR mRNA

RT-PCR analysis was used to characterize the expression of C3aR in isolated cells from human brain. The cDNAs so obtained were cloned, sequenced, and compared with the published sequence. Seven oligonucleotides were designed and used for RT-PCR analysis. The human C3aR intron/exon organization has not yet been reported, so we were not able to choose primers spanning an intron sequence. Thus, all RNA samples were treated with Rq1 DNase to eliminate contamination with genomic DNA, and it was confirmed that no PCR fragment was obtained when the RT step was omitted, when RNA was substituted with water, or when no primer was added to the PCR mix. The different combinations of C3aR primers were used in RT-PCR with total RNA extracted from control U937 (Fig. 6A) and PMA-differentiated U937 (Fig. 6B). The same strategy was used for RT-PCR with total RNA from the CB193 astrocyte cell line and fetal astrocytes (data not shown). C3aR mRNA was detected in U937, particularly after PMA differentiation, and also in CB193 and fetal astrocytes. We were not able to obtain adult astrocytes at sufficient purity in culture for analysis of mRNA expression, whereas IL-1 at 200 IU/ml decreased C3aR mRNA expression, whereas IL-1 at 200 IU/ml decreased C3aR mRNA expression by U937 was slightly (IFN-γ effect) or highly (PMA effect) up-regulated, in agreement with previous reports (34, 35). C4 mRNA expression by CB193 was up-regulated by IFN-γ and TNF-α, but was not affected by the other treatments as reported previously (36).

Expression of C3aR mRNA in astrocyte cell lines and a range of other cell lines of myeloid and nonmyeloid lineage was compared by RT-PCR (Fig. 7). C3aR mRNA was expressed by the THP1 monocyte cell line and the ECV 304 endothelial cell line, but not by B and T lymphocyte cell lines, the YT NK cell line, or the HepG2 hepatoma cell line (Fig. 7A). Southern blot analysis using a specific C3aR cDNA probe cloned from U937 confirmed the identity of the cDNA and revealed that C3aR mRNA was present at a very low level in Molt4 T lymphocyte, YT NK cell, and HepG2 cell lines (Fig. 7B). Multiple controls for false positive and false negative results were performed. Figure 7, C and D, shows RT-PCR analysis for CR2 cDNA and GAPDH cDNA on the same samples (same RT) as those used for C3aR RT-PCR. GAPDH cDNA was present in all RNA samples and at the same level; CR2 cDNA was detected only in Raji, Molt4, and CB193 astrocyte cell lines as previously described (37). The expression of CR2 mRNA by the ECV endothelial cell line demonstrated here is a new finding and merits further study.

C3aR is present in inflamed brain tissues and is expressed by myeloid and nonmyeloid cells

By RT-PCR analysis the expression of C3aR mRNA was detected in the temporal and frontal lobes, the cerebellum, and the caudate nucleus of normal control human brain (data not shown). To localize the cellular distribution of the C3aR, anti-C3aR Ab was applied to sections of normal and diseased brain. The Ab was highly specific for C3aR and was positive on frozen or formalin-fixed tissue. In all sections from normal brain, C3aR staining was

![Immunolocalization of C3aR on human adult astrocyte and astrocyte cell line CB193 cultures using peroxidase/DAB staining. Adherent cells in culture on glass coverslips (a and b, adult brain cells; c and d, CB193) were either ethanol/acetic acid permeabilized (a) or formalin fixed (b–d) and incubated with polyclonal Abs against GFAP (a) or against C3aR (b and d; affinity purified; 1.62 μg/ml). Cells were counterstained with hematoxylin (nucleus staining). a, Adult astrocytes were easily identified by their long ramified processes and strong staining for GFAP. Two large flattened cells (GFAP negative) were also present in the field (arrows) and are probably fibroblast derived cells (magnification, ×500). b, Astrocyte (large arrow) and microglia/macrophage (small arrows) present in the adult brain culture were strongly stained for C3aR. The staining was again patchy (see inset; magnification, ×1250) and restricted to some areas of the cell membrane. C3aR staining was also strongly detected on CB193 cells; magnification, ×1250). No nuclear or cytoplasmic staining of adult astrocytes or CB193 was detected using the anti-C3aR (c) CB193 negative control staining with nonimmune rabbit antiserum (IgG cut).
almost undetectable except for a very faint, but reproducible, staining on some astrocytes and a stronger staining on peripheral macrophages. However, sections of MS (Fig. 8, a–d) and acute BM (Fig. 8, e–h) brain gave a strong and specific C3aR staining. Reactive astrocytes (Fig. 8, b and e), ameboid microglia (Fig. 8c), and ramified microglia (Fig. 8d) were consistently and strongly stained for C3aR in both conditions. In MS, but not in BM, cells in the brain blood vessel wall were also strongly stained for C3aR (Fig. 8b), and these cells were identified as smooth muscle cells (pericytes). In acute MS, C3aR was expressed at high level on infiltrating perivascular macrophages, but not on infiltrating lymphocytes (data not shown). In the lumen of the vessels, erythrocytes were always C3aR negative (see Fig. 8b, inset). Infiltrating PMN in BM were also stained for C3aR (Fig. 8, f and h). At high magnification it was noted that C3aR staining on neutrophils was variable in distribution, i.e., patchy on some cells and homogeneously distributed on others (Fig. 8, f and h). Occasional perivascular macrophages present in BM sections were also stained for C3aR (Fig. 8g). Double IF staining of MS tissue sections (Fig. 9) confirmed that all cells positive for C3aR were also either GFAP positive (astroglia) or HLA class II positive (macrophage/microglia).
Discussion

Despite a substantial body of functional evidence indicating the existence of a specific receptor on some cell types for the C3a anaphylatoxin, the receptor was only conclusively identified in 1996 with the cloning of cDNA encoding the C3aR (24–26). C3aR is predicted to be a member of the seven-transmembrane-spanning receptor family, but differs from previously characterized members of the family in that the second extracellular loop is very large (172 amino acids in total). Data concerning the protein encoded by the cDNA and its tissue distribution are sparse because of the lack of reagents for immunodetection. We here describe the generation of highly specific Abs reactive with the predicted large extracellular loop of the C3aR and the use of these reagents and molecular probes to characterize the expression and molecular mass of the C3aR protein in leukocytes, glia, and brain tissue.

The presence of a C3aR on monocyte cell lines was first demonstrated by showing a specific and saturable binding of C3a to U937 cells (18, 34). Differentiation of U937 using PMA only slightly increased C3a binding, whereas differentiation with dibutyryl cAMP increased binding 7-fold. We here show, using the specific anti-C3aR Ab, that monocyte lines THP1 and U937 express C3aR and that PMA differentiation caused an up-regulation of C3aR levels. Up-regulation of C3aR was detectable only after prolonged incubation with PMA; in contrast, CR3 was up-regulated within 1 h of PMA exposure. The rapid up-regulation of CR3 is a consequence of mobilization of an intracellular pool, and the slow response of C3aR suggests that no PMA-responsive pool is present. Up-regulation of C3aR by PMA followed kinetics similar to those of C3aR, but the increase was much greater, up to 10-fold.

IFN-γ in our hands was the most powerful regulator of C3aR expression by two different monocyte cell lines. Even at 10 IU/ml, IFN-γ caused a 2-fold increase in C3aR expression on U937. The effect of IFN-γ on our hands was the most powerful regulator of C3aR expression by two different monocyte cell lines. Even at 10 IU/ml, IFN-γ caused a 2-fold increase in C3aR expression on U937. The effect of IFN-γ on our hands was the most powerful regulator of C3aR expression by two different monocyte cell lines. Even at 10 IU/ml, IFN-γ caused a 2-fold increase in C3aR expression on U937. The effect of IFN-γ on our hands was the most powerful regulator of C3aR expression by two different monocyte cell lines. Even at 10 IU/ml, IFN-γ caused a 2-fold increase in C3aR expression on U937.
that expression of C5aR in the inflamed brain is important in perpetuation of the inflammatory response. The primary aim in generating reagents for detection of C3aR was to ascertain whether it follows a similar pattern of expression in brain cells and tissue. In the original descriptions of cloning, the 2.2-kb C3aR mRNA was detected by Northern analysis in human brain tissue (24–26); in the present report we confirm this finding. However, no data were available on the cell types expressing C3aR in brain. Staining with the specific anti-C3aR Ab demonstrated that astrocyte cell lines and primary fetal and adult astrocytes expressed C3aR in vitro, with the more differentiated of the cell lines and adult astrocytes expressing more than the undifferentiated cell line and fetal astrocytes. To further confirm that the receptor expressed by astrocytes was identical with that cloned from leukocyte cell lines we used a PCR strategy to clone and sequence astrocyte C3aR cDNA. The majority of the astrocyte C3aR cDNA sequence (residues 369–1433) was obtained in this manner and was 100% identical with the published sequence obtained from HL60 cells (24).

Although all cells in astrocyte cultures expressed C3aR, the distribution on individual cells was not homogeneous; C3aR staining was detected on ramified microglia, rare perivascular macrophages, and a few astrocytes (data not shown). b, Acute MS: C3aR-positive staining on the majority of reactive astrocytes as well as on cells in the blood vessel wall. Note that erythrocytes were all negative for C3aR. Higher magnification (inset) revealed that the C3aR staining in blood vessel wall was on pericytes (smooth muscle cells). c, Acute MS: a cluster of ameboid microglia rich in vacuoles was strongly stained for C3aR. d, Chronic MS: ramified microglia were C3aR positive, and here the staining was confirmed on frozen tissue sections. e and h, BM: The expression of C3aR was dramatically elevated in BM brains. Astrocytes (e and inset), microglia (not shown) and infiltrating cells, macrophages (g), and neutrophils (f and h) were all strongly stained for C3aR. Not all neutrophils in the meninges expressed C3aR at a high level. h, C3aR staining on the membrane of an infiltrating neutrophil is clustered. No C3aR staining was detected on the blood vessel wall in BM. All photographs were taken at ×500, except for b, d, e, and f insets (×1250).
FIGURE 9. Double IF staining of MS tissue sections for C3aR. Frozen MS brain tissue sections were double stained for C3aR (FITC, rabbit anti-C3aR peptide) and either for astrocyte cell marker (GFAP, mouse Ab) or microglia cell marker (HLA class II, mouse LN3 clone). Specific binding of the primary Ab was detected using FITC-conjugated goat anti-rabbit Ab (SeraLab) and rhodamine-conjugated goat anti-mouse Ab (Sigma). a and b, MS sections stained for C3aR and GFAP. c and d, MS sections stained with two irrelevant Abs. e and f, MS sections stained for C3aR and HLA class II. All GFAP-positive cells expressed C3aR. HLA class II-positive cells were mainly identified as microglia and infiltrating macrophages, and they all expressed C3aR. No C3aR-positive cells were detected that did not also express markers for the astrocyte or the microglia/macrophage population.

proteins are colocalized. Several other membrane proteins stained using an identical protocol gave a homogeneous distribution pattern on astrocytes, eliminating the possibility that this unusual distribution pattern of C3aR and C5aR is an artifact of the staining procedure. We suggest that the enrichment of the receptor at specific areas on the cell membrane is of functional significance, perhaps enhancing the capacity of the cells to respond to the anaphylatoxins. It will be interesting to examine whether the distribution of the anaphylatoxin receptors alters when cells are stimulated possibly enhancing the capacity of the cells to respond to the anaphylatoxins.

Microglia derived from fetal and adult brain were also strongly stained for C3aR (Fig. 4b). This finding was anticipated because microglia are derived from the monocyte/macrophage lineage (38) and strongly express C5aR (16). The demonstration of C3aR expression by human microglia also supports the recent report that C3a induces calcium fluxes in cultured murine microglia (29). Oligodendrocytes and neurons in culture were always negative for C3aR.

The molecular mass of expressed C3aR has not previously been determined. The cDNA sequence predicts a mature protein of 482 amino acids and a molecular mass of 54 kDa; however, the sequence contains two putative N-glycosylation sites. Immunoprecipitation of C3aR from leukocyte lines or from astrocyte lines followed by Western blotting demonstrated that the protein from both sources had a molecular mass of 65 kDa, implying that the C3aR is heavily glycosylated in both cell types (Fig. 5C). Although all immunoprecipitates were from whole cells, the predicted 54-kDa unglycosylated C3aR precursor was never detected. We are currently confirming the above data by growing cells in tunicamycin to inhibit glycosylation before immunoprecipitation. Experiments are also underway to define the molecular mass of C3aR from eosinophils, neutrophils, monocytes, and primary astrocytes. The principal difficulty in these experiments is to obtain sufficient numbers of cells in sufficient purity for use in the immunoprecipitation protocol.

Expression of C3aR was confirmed for the various cell lines and primary cells at the mRNA level by RT-PCR. All cells positive by IF were also positive by RT-PCR (Fig. 7). The endothelial cell line ECV304, untested in IF, was also positive, whereas the hepatoma cell line HepG2, also untested in IF, was negative. The absence of detectable message for C3aR in HepG2 cells is interesting in that this cell line is reported to express the C5aR (5, 13); it may thus represent the unusual occurrence of expression of one anaphylatoxin receptor without the other. To confirm specificity and increase sensitivity, a combination of RT-PCR and Southern blotting was used. With this protocol, C3aR message was detected at very low levels in the various lymphocyte cell lines and in HepG2 (Fig. 7).

Given the above data on glial cell expression of C3aR and the presence of C3aR message in brain tissue (24–26), it was expected that C3aR would be detected by immunohistochemistry in brain. However, normal brain tissue was almost completely negative for C3aR expression, although the Ab readily detected C3aR in other normal tissues (lung, liver, and adrenal gland) subjected to a similar processing protocol (data not included). In marked contrast, inflamed brain tissue (MS or meningitis) was strongly positive for C3aR (Figs. 8 and 9). This finding is, again, very similar to that obtained for C5aR, which was barely detectable in normal brain but was highly expressed in inflammation (16). The distribution of C3aR expression differs between the two conditions chosen for study. In both, astrocytes and microglia in the areas of pathology and infiltrating phagocytes (macrophages in MS, neutrophils in meningitis) express C3aR. In MS, a strong perivascular staining was observed, which was not present in meningitis tissue, that appeared to be associated with pericytes. The functional significance of expression of C3aR by pericytes in the vessel wall is uncertain, but it does suggest that the anaphylatoxins might influence vessel tone or permeability in MS brain. Elevated expression of anaphylatoxin receptors in CNS tissue appears to be a hallmark of inflammatory processes and is probably associated with intrathecal complement activation and local generation of the anaphylatoxins (27, 39). The expressed receptors may then contribute to an autocrine pathway, activating glia and recruiting myeloid and nonmyeloid cells into the brain tissue (38). This scenario is supported by the in vitro observation that C3a and C5a stimulate and also induce chemotaxis of astrocytes and microglia (14, 28, 29, 40). Monocyte/macrophage expression of cytokines, chemokines, and other immune molecules is regulated by C3a and C5a (3, 12, 19, 41); we are now testing whether C3a and/or C5a have similar effects on astrocytes and microglia in culture.

To address the roles of the anaphylatoxins and their receptors in CNS inflammation it will be necessary to use models in which the
receptors and their ligands can be manipulated. We have established rodent models of demyelination and ischemia (both conditions characterized by a severe brain inflammation with complement activation and genesis of anaphylatoxins), and we plan to block specifically the effects of both anaphylatoxins using specific antagonists and neutralizing Abs for ligands and receptors. Mouse and rat C5aR have been cloned, and recently, mouse C3aR has been cloned from a brain cDNA library (42–44). The reagents necessary for receptor blockade in rodents are currently being generated.

Acknowledgments

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References