# Deletion of the Complement Anaphylatoxin C3a Receptor Attenuates, Whereas Ectopic Expression of C3a in the Brain Exacerbates, Experimental Autoimmune Encephalomyelitis[1]

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**The C3aR is expressed throughout the CNS and is increased in expression on glial cells during CNS inflammation. However, the role that C3a and the C3aR play in chronic inflammation, such as in the demyelinating disease experimental autoimmune encephalomyelitis (EAE), remains unclear. We show in this study that deletion of the C3aR is protective in myelin oligodendrocyte glycoprotein-induced EAE in C57BL/6 mice. C3aR-deficient ($C3aR^{-/-}$) mice had a significantly attenuated course of EAE compared with control mice during the chronic phase of the disease. Immunohistochemical analysis demonstrated modestly reduced macrophage and T cell infiltration in the spinal cords of $C3aR^{-/-}$ mice. To examine the role of C3a in EAE, we developed a transgenic mouse that expresses C3a exclusively in the CNS using the glial fibrillary acidic protein (GFAP) promoter. We observed that C3a/GFAP mice had exacerbated EAE during the chronic phase of the disease, with significant mortality compared with nontransgenic littermates. C3a/GFAP mice had massive meningeal and perivascular infiltration of macrophages and $CD4^+$ T cells. These studies indicate that C3a may contribute to the pathogenesis of demyelinating disease by directly or indirectly chemoattracting encephalitogenic cells to the CNS.** ***The Journal of Immunology*****, 2004, 173: 4708–4714.**

The anaphylatoxin C3a is a pleiotropic molecule with diverse functions in immune responses. C3a mediates these functions upon binding to the C3aR expressed on smooth muscle, epithelial, and endothelial cells (1–3) and most myeloid cells, including hemopoietic stem cells, neutrophils, monocytes, macrophages, mast cells, basophils, eosinophils, T and B cells, and monocyte-derived dendritic cells (4–8). In the CNS, the C3aR is expressed on microglia, astrocytes, and neurons (9–13). The C3aR is a pertussis toxin-sensitive, G-protein-coupled ($G_{\alpha 16}$ and $G_{\alpha 12/13}$), seven-transmembrane-spanning receptor (2, 14–19). Binding of C3a to the C3aR causes $Ca^{2+}$ mobilization (14, 17, 20), and signaling appears to occur through MAPK and PLC pathways (21).

C3a mediates both proinflammatory and anti-inflammatory activities on binding the C3aR. Proinflammatory activities include chemoattraction of inflammatory cells, including neutrophils, mast cells, and basophils; degranulation of mast cells and basophils (22, 23); cytokine and chemokine synthesis (3, 24, 25); inducing the production of reactive oxygen species (26–28); and increasing the expression of adhesion molecules (29). In experimental allergic asthma, the absence of the C3aR abrogated or severely reduced lung and airway recruitment of eosinophils, Th2 activation and recruitment to the lung, and IgE and IgG1 secretion by B cells (30). Receptor-deficient mice also had reduced levels of IL-4, IL-5, and IL-13 in their lungs, suggesting that the C3aR plays an important role in airway inflammation in this disease. Conversely, there is substantial evidence that C3a mediates anti-inflammatory functions as well. C3a suppresses LPS-induced TNF-$\alpha$, IL-1$\beta$, and IL-6 secretion by human PBMC in vitro and modulates the production of anti-inflammatory hormones (31–36). Mice deficient in C3aR are more sensitive to LPS-induced lethality, and after LPS challenge, plasma concentrations of IL-1$\beta$ (37), suggesting a protective role for the C3aR in modulating proinflammatory cytokine production.

Increased expression of the C3aR by resident CNS cells and, in some cases, infiltrating cells has been reported in experimental models of bacterial meningitis (9), focal ischemia (11, 12), and Alzheimer's disease (38). In demyelinating disease, C3aR expression is increased in experimental autoimmune encephalomyelitis (EAE)[3] and multiple sclerosis (MS) (9, 10) In an effort to determine the role of C3a and the C3aR in EAE, we performed myelin oligodendrocyte glycoprotein (MOG)-induced EAE in mice deficient for the C3aR ($C3aR^{-/-}$) and in transgenic mice with astrocyte-specific expression of C3a (C3a/glial fibrillary acidic protein (GFAP)). Mice lacking a functional receptor for C3a showed attenuated disease severity in MOG-induced EAE, whereas C3a/GFAP transgenic mice had markedly worse disease. These data indicate that C3a contributes to the pathogenesis of EAE and suggests that targeting C3a may have therapeutic value in demyelinating disease.

## Materials and Methods

### *$C3aR^{-/-}$ mice*

$C3aR^{-/-}$ mice have been described previously (37) and were crossed eight generations to the C57BL/6 background. These animals produce no C3aR mRNA or protein. $C3aR^{-/-}$ mice were frequently screened by PCR to

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[3] Abbreviations used in this paper: EAE, experimental autoimmune encephalomyelitis; CDI, cumulative disease index; GFAP, glial fibrillary acidic protein; hGH, human growth hormone; MOG, myelin oligodendrocyte glycoprotein; MS, multiple sclerosis; RPA, RNase protection assay.



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Table I. *EAE clinical features in control and C3aR$^{-/-}$ mice*

| Experimental Group | Incidence (%) | Onset (days postimmunization) | CDI[a] | Mortality |
|---|---|---|---|---|
| Control ($n = 16$) | 100 | 15 | 66.7 | None |
| C3aR$^{-/-}$ ($n = 15$) | 100 | 16 | 48.2[b] | None |

[a] CDI is the average of the sum of the daily clinical scores observed between days 1 and 30. Shown are the mean for each group.
[b] Significantly less severe disease compared with control mice ($p = 0.04$, by ANOVA).

verify the absence of the C3aR, as previously described (37). These mice develop normally, are fertile, and have normal populations of leukocytes.

### *Generation of C3a/GFAP transgenic mice*

A cDNA construct encoding the murine C3a sequence (using the murine IL-6 signal sequence for proper secretion) was generated by PCR. The construct was subcloned into the *Not*I site in the pGF.GH construct, 3′ to the GFAP promoter and 5′ to the human growth hormone (hGH) polyadenylation sequence as previously described (39). Due to cloning constraints the N-terminal serine of C3a was converted to glutamine. All PCR products and the final construct were sequenced to verify that the correct sequences were used in making the final construct. The C3a/GFAP construct was microinjected into one-cell C57BL/6J mouse eggs by the UAB Transgenic Animal/ES Cell facility. Founders were identified by PCR analysis of tail-derived DNA using 5′-C3a-specific (mC3a.1, TCA GTA CAG TTG ATG GAA AGA) and 3′-hGH-specific (hGH.1, TGG GCA TGG AGT GGC AAC TT) primers. The PCR protocol was as follows: 30-s denaturation at 94°C, 1 min annealing at 56°C, and 45-s elongation at 72°C for 35 cycles. Three founder lines designated 5, 7, and 10 were identified. CNS-specific expression of the C3a transgene was assessed using RNA isolated from organs (brain, liver, lung, spleen, gut, kidney, heart, and bladder) from C3a transgenic mice and their negative littermates using the RNAgent kit (Promega, Madison, WI). cDNA was produced from 2 $\mu$g total RNA by RT using oligo-dT15 primers and Moloney murine leukemia virus reverse transcriptase (Promega). Subsequent PCR amplification of the generated cDNA was performed following the protocol described above.

### *C3a transgene expression by primary astrocyte cultures*

Primary astrocyte-rich cultures were obtained from cerebral hemispheres of neonatal C3a/GFAP transgenic mice and their negative littermates as previously described (40). Confluent, cortical astrocytes cultured in 75-cm$^2$ flasks were used after 14–20 days for RT-PCR and immunofluorescence studies. Astrocyte cultures were treated with 100 ng/ml LPS and 10 $\mu$g/ml brefeldin A for 6 h at 37°C. The cultures were washed in PBS, fixed in 4% formaldehyde, and permeabilized with 0.1% Triton/PBS. Immunodetection of C3a-producing astrocytes was performed using a polyclonal rabbit anti-mC3a Ab (1/100; Research Genetics, Huntsville, AL) and an Alexa Fluor goat anti-rabbit IgG secondary Ab (1/200; Molecular Probes, Eugene, OR). Double staining for GFAP, an astrocyte marker, using Alexa Fluor donkey anti-goat (1/50; Santa Cruz Biotechnology, Santa Cruz, CA) was performed on the same culture slides. C3a/GFAP mice appeared phenotypically normal and had no CNS or other morphological abnormalities under both micro- and macroscopic examination (data not shown). We did not observe spontaneous development of inflammation in the C3a/GFAP at any time.

### *Generation of C3a/GFAP×C3aR$^{-/-}$ mice*

C3a/GFAP mice were crossed with C3aR$^{-/-}$ mice. Heterozygote mice were crossed with C3aR$^{-/-}$ mice, and the resulting transgenic C3aR$^{-/-}$ offspring were selected as breeders. All generations of these mice were screened using the PCR protocols described above for C3aR$^{-/-}$ and C3a/GFAP mice.

### *EAE induction and evaluation*

C3a/GFAP transgenic, C3aR$^{-/-}$, or C3a/GFAP × C3aR$^{-/-}$ mice were compared with and simultaneously immunized along with wild-type C57BL/6 mice. All transgenic or mutant mice were screened by PCR to verify genotype before the start of EAE studies. All mice used in this study were between 8 and 12 wk of age at the time of the first immunization. Mice were immunized with MOG peptide 35–55 (Biosynthesis, Lewisville, TX). Mice were injected s.c. on days 0 and 7 with 150 $\mu$g of peptide emulsified with 50 $\mu$g of *Mycobacterium tuberculosis* in IFA. In addition, on days 0 and 2 postimmunization, mice were given pertussis toxin (500 ng) i.p. Clinical signs of EAE were assessed daily for 30 days using a standard scale of 0–6 as follows: 0, no clinical signs; 1, loss of tail tone; 2, flaccid tail; 3, incomplete paralysis of one or two hind legs; 4, complete hind limb paralysis; 5, moribund requiring killing the animals; and 6, death. Mice were considered to have EAE when they presented with a score of 2 or more for at least 2 consecutive days. For all groups of mice, a cumulative disease index score (CDI) was calculated based on the sum of the daily average of the clinical scores. All mouse studies were performed with approval of the University of Alabama institutional animal care and use committee.

### *Histological assessment and immunohistochemistry*

On days 24–30 mice were killed by $CO_2$ inhalation, and brains and spinal cords were removed, flash-frozen, and kept at −80°C until needed. Spinal cords were cut into 9-$\mu$m sections, and immunohistochemistry was performed using the Vectastain avidin-biotin kit (Vector Laboratories, Burlingame, CA). Ethanol- or acetone-fixed sections were incubated for 30 min with rat anti-mouse CD11b, CD3, CD4, or CD8 (1/100; BD Pharmingen, San Diego, CA). Biotin-conjugated goat anti-rat IgG was then applied (1/100; Jackson ImmunoResearch, West Grove, PA). The sections were treated with avidin-peroxidase for 30 min before being developed with a diaminobenzidine detection kit (Vector Laboratories).

### *RNase protection assay (RPA)*

Total RNA was isolated from spinal cord tissue using the RNAgents RNA extraction kit (Promega) according to the manufacturer's instructions. Nonradioactive RPA probe transcription was performed using Non-Rad In Vitro Transcription Kit (BD Biosciences, San Diego, CA) according to manufacturer's instructions. Briefly, 20 $\mu$g of total RNA was incubated with various biotin-labeled BD RiboQuant MultiProbe Sets (mCk-2b, mCk-3b, mCk-5c, and mAPO-1; BD Biosciences, San Diego, CA) overnight. Protected RNA probes were resolved on a 4.75% acrylamide gel. Probes were transferred to a positively charged nylon membrane and detected using the Non-Rad Detection Kit (BD Biosciences) according to manufacturer's instructions. Autoradiographs were scanned and analyzed using the National Institutes of Health ImageJ program.

### *Statistical analysis*

Statistical analysis was performed using the StatView software package (SAS Institute, Cary, NC). Statistical significance of all results was assessed using Student's $t$ test or ANOVA. A value of $p < 0.05$ was considered significant.

## Results

### *Deletion of the C3aR attenuates MOG-induced EAE*

To determine whether the C3aR is critical to the initiation and progression of EAE, we immunized C3aR$^{-/-}$ and control

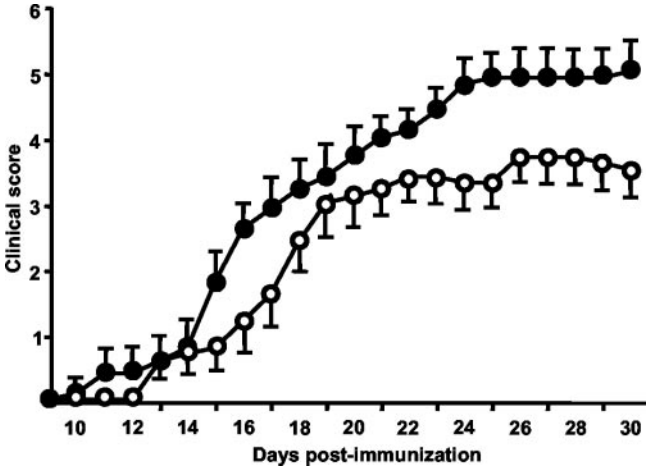


**FIGURE 1.** Clinical course of MOG-induced EAE in control and C3aR$^{-/-}$ mice. EAE was induced in control ($n = 16$; ●) and C3aR$^{-/-}$ ($n = 15$; ○) mice and scored as described in *Materials and Methods* for 30 days. The results shown are an average obtained from four separate experiments. The course of EAE in C3aR$^{-/-}$ mice was significantly less severe ($p < 0.04$, by ANOVA) than that in control mice.

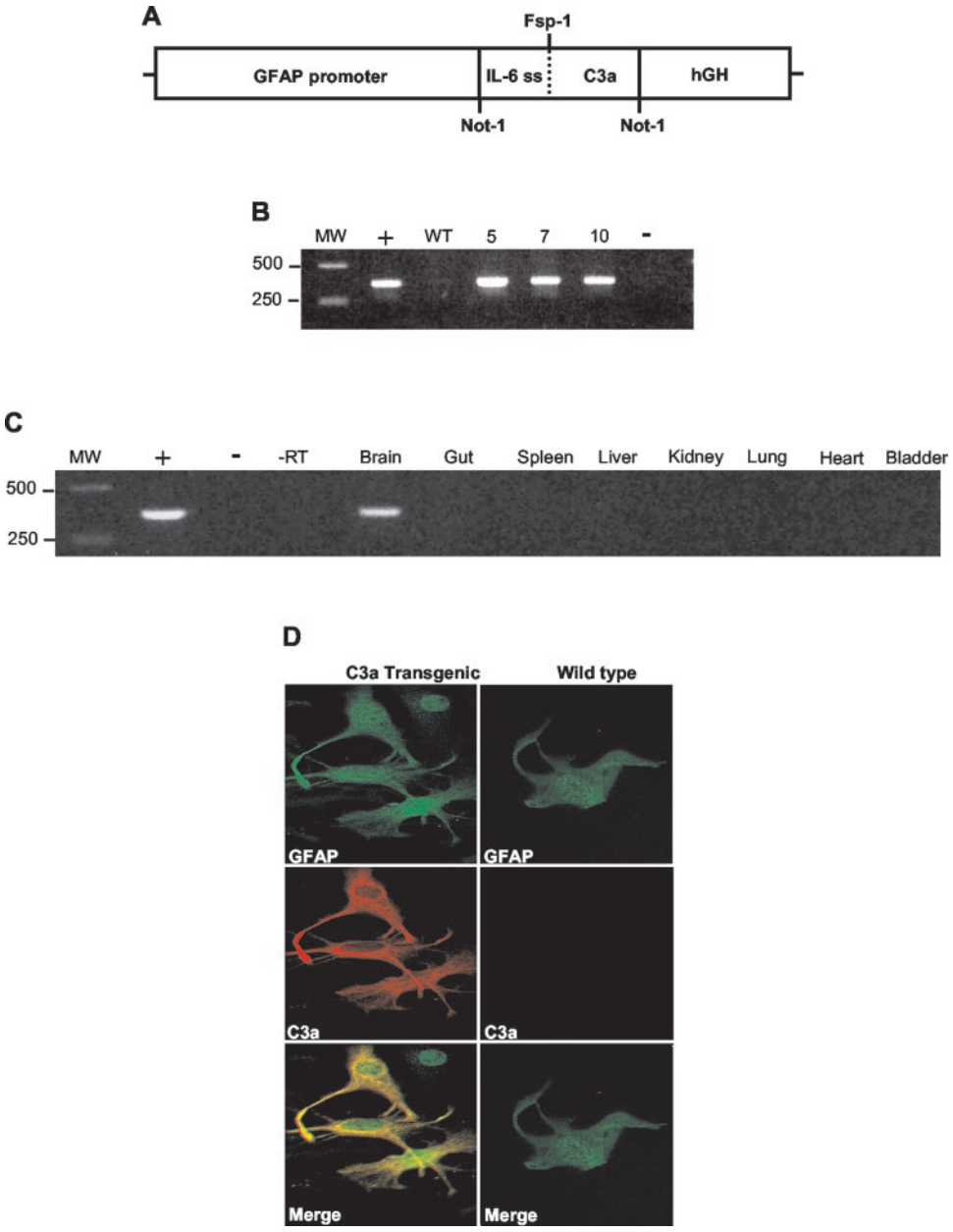


**FIGURE 2.** Characterization of C3a/GFAP mice. *A*, Schematic diagram of the C3a/GFAP construct. cDNA encoding murine C3a with an IL-6 signal sequence (IL-6 ss) were subcloned into the *Not*I site in the GFAP promoter construct. *B*, Three C3a/GFAP founder lines were identified (5, 7, and 10) by PCR analysis of tail DNA using the primers described in *Materials and Method*s. MW, molecular weight markers of 250 and 500 bp are shown; (+), PCR using the C3a transgene construct; (−), PCR using the DNA from a nontransgenic littermate. *C*, RT-PCR analysis of mRNA isolated from brain, gut, spleen, liver, kidney, lung, heart, and bladder demonstrates brain-specific expression of the C3a transgene. Shown are representative results using tissues from a line 10 C3a/GFAP mouse. Similar results were obtained using tissues from line 7 mice. *D*, Astrocytes isolated from C3a/GFAP and control mice were treated with LPS and brefeldin A for 6 h to block protein secretion. The astrocytes were then analyzed by immunofluorescence using anti-GFAP (green) and rabbit anti-murine C3a (red) Abs. Colocalization shows production of the C3a transgene product in C3a/GFAP astrocytes.

C57BL/6 mice with $MOG_{35–55}$ peptide and compared the two groups for disease onset and severity. Control and $C3aR^{-/-}$ mice had a 100% disease incidence and developed disease at approximately the same time (15.2 vs 16.3 days, respectively; Table I). However, ~3 wk postimmunization, clinical signs of disease in $C3aR^{-/-}$ mice reached a plateau and did not progress to the same level of severity as that seen for control mice (Fig. 1). Overall, the CDI was lower in $C3aR^{-/-}$ mice compared with control mice (48.2 vs 66.7), and the difference in disease severity was significantly less ($p = 0.04$, by ANOVA). There was a modest reduction in cellular infiltration and demyelination in the spinal cords of $C3aR^{-/-}$ mice compared with control mice (data not shown). T cells from $C3aR^{-/-}$ mice proliferated comparably to receptor-sufficient controls on in vitro stimulation with $MOG_{35–55}$ peptide, suggesting that the absence of the C3aR on T cells does not alter their encephalitogenic-inducing capacity (data not shown).

### *Characterization of C3a/GFAP mice*

The modest attenuation of EAE in the $C3aR^{-/-}$ mice coupled with reduced cellular infiltration suggested that the contributions of C3a and the C3aR to EAE are primarily chemoattraction of inflammatory cells to the CNS. To test this possibility, we used C3a/GFAP transgenic mice developed in our laboratory. To generate C3a/GFAP transgenic mice, cDNA encoding murine C3a was placed under the transcriptional control of the astrocyte-specific GFAP promoter (Fig. 2*A*). The murine IL-6 signal sequence was subcloned 5′ to the C3a sequence to allow for proper secretion of the transgene product. Three founder lines (5, 7, and 10) were positive for the presence of C3a/GFAP DNA (Fig. 2*B*) and were used to establish separate transgenic lines. Line 5 bred poorly and was not used in additional studies. Lines 7 and 10 expressed C3a exclusively in the CNS, as determined by RT-PCR. A representative example of tissue-specific transgene expression using tissue from brain, gut, spleen, liver, kidney, lung, heart, and bladder is shown using mRNA from a C3a/GFAP mouse from line 10 (Fig. 2*C*). To determine whether astrocytes from C3a/GFAP mice produced C3a, we stimulated primary astrocyte cultures from control and C3a/GFAP mice with LPS and brefeldin A for 6 h. Astrocytes were then fixed and assessed for the presence of C3a by immunofluorescence using rabbit anti-C3a Abs. C3a was readily detected in

LPS-stimulated astrocytes from C3a/GFAP mice, but not from control mice (Fig. 2*D*). No C3a staining was detected in transgenic astrocytes treated only with brefeldin A (data not shown).

### *C3a/GFAP mice develop severe EAE*

In preliminary EAE experiments, we observed that the clinical disease phenotype of C3a/GFAP mouse lines 7 and 10 were similar. C3a/GFAP line 7 was bred to homozygosity, as assessed by repeated out-crossing to wild-type mice, and was used for all subsequent EAE experiments. C3a/GFAP mice and nontransgenic littermates had similar onset and initial progression of EAE, and the incidence of disease was 100% in both groups of mice (Fig. 3 and Table II). However, at ~3 wk postimmunization, disease severity increased significantly ($p < 0.05$) in C3a/GFAP mice, but not in control mice (Fig. 3). The CDI of C3a/GFAP mice was markedly higher than that of control mice (59.8 vs 40.8). In addition to more severe clinical disease, 60% of the C3a/GFAP mice died from EAE between days 22 and 30, with 50% of mice dying before day 25. Only 16% of control mice died in the same time period (Fig. 4).

To ensure that the severe EAE seen in the C3a/GFAP mice was not an artifact due to the location of transgene insertion, we crossed C3a/GFAP mice with C3aR$^{-/-}$ mice. We then induced EAE in C3a/GFAP×C3aR$^{-/-}$ and control mice and scored them for 30 days (Fig. 5). The C3a/GFAP×C3aR$^{-/-}$ mice developed EAE with significantly reduced severity through much of the chronic phase of the disease ($p < 0.05$; days 17–25, 29, and 30), comparable to C3aR$^{-/-}$ mice (see Fig. 1). None of the C3a/GFAP×C3aR$^{-/-}$ mice displayed a course of EAE worse than control mice or died from EAE. These results are consistent with the C3a transgene product using the endogenous C3aR to induce severe EAE.

### *EAE in C3a/GFAP mice is characterized by massive cellular infiltrate, but little difference in demyelination*

We performed immunohistochemistry with Abs to CD11b, CD3, CD4, and CD8 to examine differences in macrophage and T cell infiltration in control and C3a/GFAP mice. We observed perivascular and meningeal infiltration of macrophages and T cells characteristic of MOG-induced EAE in control mice (Fig. 6, *A* and *B*). In contrast, spinal cord sections from C3a/GFAP mice were often completely surrounded by infiltrating macrophages with accompanying heavy perivascular infiltration (Fig. 6*C*, see arrows). Infiltrating cells were observed throughout both the white and gray matter of the spinal cord. In addition, there was a markedly elevated T cell infiltration in the spinal cords of C3a/GFAP mice as assessed by CD3 immunostaining (Fig. 6*D*, see arrows). The majority of these cells were CD4$^+$ T cells (Fig. 6*E*) with relatively few infiltrating CD8$^+$ T cells (data not shown). Control IgG Ab demonstrated low background staining (Fig. 6*F*). Demyelination, as assessed by toluidine blue staining of paraformaldehyde-fixed spinal cord sections, was not markedly different between control and C3a/GFAP mice with similar clinical disease. We observed scattered focal regions of necrosis in the white matter of C3a/GFAP mice with EAE more frequently than in control mice (data not shown).

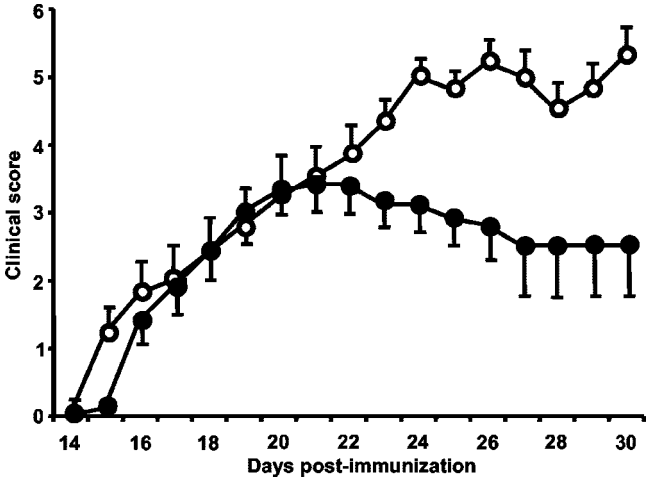


**FIGURE 3.** Clinical course of MOG-induced EAE in control and C3a/GFAP mice. EAE was induced in control ($n = 12$; ●) and C3a/GFAP ($n = 15$; ○) mice and scored for 30 days. Results shown are an average obtained from five separate experiments. The course of EAE in C3a/GFAP mice was significantly more severe ($p < 0.02$, by ANOVA) than that in control mice.

Table II. *EAE clinical features in control and C3a/GFAP and C3a/C3aR$^{-/-}$ mice*

| Experimental Group | Incidence (%) | Onset (days postimmunization) | CDI[a] | Mortality |
|---|---|---|---|---|
| Control ($n = 12$) | 100 | 18 | 40.8 | 2/12 |
| C3a/GFAP ($n = 15$) | 100 | 18 | 59.8[b] | 9/15 |
| Control ($n = 6$) | 100 | 18 | 47.5 | None |
| C3a/C3aR$^{-/-}$ ($n = 6$) | 100 | 19 | 35.2 | None |

[a] CDI is the average of the sum of the daily clinical scores observed between days 1 and 30. Shown are the mean for each group.

[b] Significantly worse disease compared with control mice ($p = 0.02$, by ANOVA).

## Discussion

In the present study we show that C3aR and its ligand, C3a, contribute to the pathogenesis of EAE. Deletion of C3aR attenuated MOG-induced EAE in the effector phase of the disease, but did not alter the onset or initial progression of EAE. This result suggests that the C3aR does not modulate Ag-specific T cell functions early in the development of EAE despite its expression on T cells (5, 7). The fact that we did not observe any difference in the proliferative capacity of splenic T cells between control and C3aR$^{-/-}$ mice with EAE supports this argument. The attenuated EAE seen in C3aR$^{-/-}$ mice could be due to an altered cytokine profile. To address this possibility, we performed RPAs for a panel of cytokines. We found modest elevation of mRNA for TGF-$\beta$ and IL-6 (2- to 3-fold), and substantially higher elevation of mRNA for MCP-1, IP-10, RANTES, and IL-1$\beta$ in C3aR$^{-/-}$ mice compared with control mice (>10-fold; data not shown). IL-1$\beta$ mRNA, in particular, was elevated, as much as 90-fold in some experiments. Despite these increases in mRNA levels, there was no corresponding increase in IL-1$\beta$ protein levels in the spinal cords of C3aR$^{-/-}$ mice or for several other cytokines, as assessed by ELISA (data not shown). This suggests that the attenuated EAE in the C3aR$^{-/-}$ mice is not due to a dramatic shift in expression of cytokines in the spinal cord. Interestingly, IL-1$\beta$ serum levels are elevated in C3aR$^{-/-}$ mice during endotoxic shock (37). These results combined with those of our EAE studies suggest an important role for the C3aR in modulating IL-1$\beta$ expression in vivo. C3a alone does not modulate proinflammatory cytokine expression (IL-1$\beta$, IL-6, and TNF-$\alpha$) in PBMCs and B cells (31–34). However, stimulation of either PBMCs or B cells with bacterial cell by-products in the presence of C3a induces cytokine mRNA and protein production. Thus, C3a may serve as an important cofactor in modulating proinflammatory cytokine expression in immune responses.

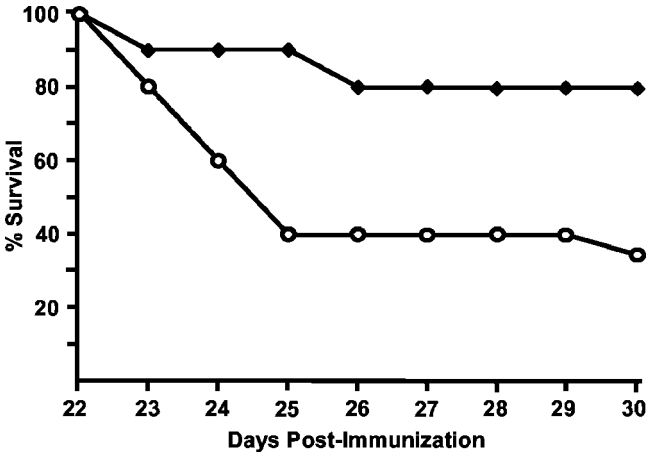


**FIGURE 4.** Survival of control and C3a/GFAP mice after induction of EAE. The control (♦) and C3a/GFAP (●) mice were monitored for survival after induction of EAE. Shown is the percent survival of mice from days 22–30. No mouse died before day 22.

We developed C3a/GFAP mice to assess alterations in CNS pathophysiology as a result of endogenous expression of C3a under inflammatory conditions. Although we anticipated that C3a/GFAP mice would develop exacerbated EAE, we did not expect the frequently fatal phenotype we observed. Interestingly, C3a/GFAP mice progressed to severe EAE at the same time that $C3aR^{-/-}$ mice showed attenuated disease, ~3 wk postimmunization. C3a/GFAP mice presented with a remarkably higher mortality rate than control mice (60 vs 16%). In most cases, the progression to death was rapid (overnight) and occurred in animals with modest disease (clinical scores of 2–3) rather than in moribund animals. The average clinical score of C3a/GFAP mice that survived until day 30 was not significantly different from that of control mice. This raises the possibility that greater disease penetrance in some C3a/GFAP mice may account for the higher mortality during EAE. We did not observe any significant differences in cytokine/chemokine gene expression in mRNA prepared from spinal cord of C3a/GFAP mice compared with control mice. Given the severe disease phenotype in the C3a/GFAP mice, this finding was somewhat unexpected, but perhaps not too surprising given

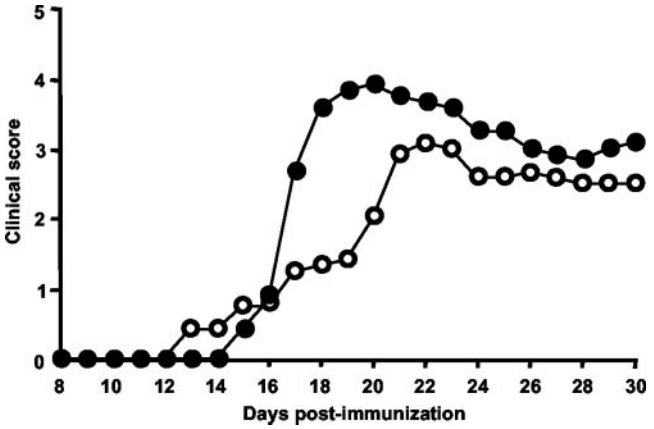


**FIGURE 5.** Clinical course of MOG-induced EAE in control and C3a/GFAP×$C3aR^{-/-}$ mice. EAE was induced in control ($n = 6$; ●) and C3a/GFAP×$C3aR^{-/-}$ ($n = 6$; ○) mice and scored for 30 days. Results shown are an average obtained from one experiment. The course of EAE in C3a/GFAP×$C3aR^{-/-}$ mice was significantly less severe ($p < 0.05$) throughout most of the chronic phase of the disease (days 17–25) than that in control mice.

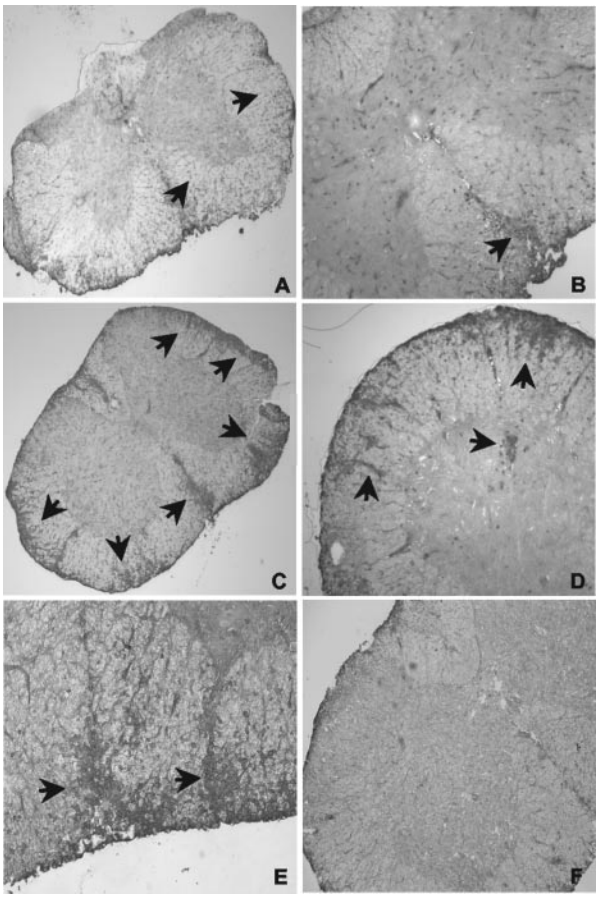


**FIGURE 6.** Increased infiltration of macrophages and T cells in the spinal cords of C3a/GFAP mice compared with control mice. *A*, Immunohistochemistry using rat anti-mouse CD11b Ab with diaminobenzidine amplification of a representative control mouse spinal cord section. *B*, Immunohistochemistry using a rat anti-mouse CD3 Ab on a representative control mouse spinal cord section. *C*, CD11b staining in the spinal cord of a C3a/GFAP mouse. Note the heavy meningeal and perivascular infiltrate. *D*, CD3 staining in the spinal cord of a C3a/GFAP mouse. Note the heavy meningeal and perivascular infiltrate. *E*, CD4 staining in the spinal cord of a C3a/GFAP mouse. The T cell infiltrate in these mice was predominantly CD4 as opposed to CD8. *F*, Control staining using an anti-rat IgG Ab as the primary Ab shows low background staining. All sections were obtained at 30 days postimmunization. Arrows point to regions of heavy cellular infiltrate. Original magnification: *A–D*, ×10; *E* and *F*, ×20.

that our RPA analysis was on the subset of C3a/GFAP mice that survived to day 30 and had clinical scores similar to those of control mice. We cannot preclude a significant role for proinflammatory cytokines and other mediators in contributing to the severe EAE and higher mortality seen in C3a/GFAP mice. Analyzing cytokine levels by RPA at earlier time points may prove informative, particularly if a pattern of cytokine expression that clearly distinguishes between chronic vs fatal disease in C3a/GFAP mice is observed.

One possible contribution of the C3aR/C3a receptor-ligand pair to the maintenance of chronic EAE is chemoattraction of encephalitogenic cells into the CNS. The most striking feature of EAE in C3a/GFAP mice is the massive infiltration of macrophages and $CD4^+$ T cells in and around the spinal cord. The meninges and peripheral white matter in some C3a/GFAP mice were essentially overrun with both cell types (see Fig. 5). In addition, there was markedly higher perivascular infiltration in C3a/GFAP mice in the white and gray matter compared with that in control mice. $CD4^+$

T cells were the predominant lymphocyte infiltrating the spinal cord of C3a/GFAP mice, although we observed a few $CD8^+$ T cells scattered throughout the parenchyma. It is not clear whether the proportion of $CD4^+$ vs $CD8^+$ T cells in the CNS of the C3a/GFAP mice is a function of differential C3aR expression on T cell subpopulations during EAE. Although it is possible that CNS-derived C3a is directly chemoattracting T cells (or macrophages), it is also likely that C3a contributes to the induction and expression of other chemoattractants as well. Several studies have demonstrated that C3a induces the expression of cytokines, including IL-1$\beta$, IL-6, IL-8, MCP-1, and RANTES, by astroglioma cell lines and endothelial cells (3, 24, 25). In addition, C3a may cause degranulation of brain mast cells, potentially leading to the release of a variety of inflammatory mediators, including cytokines and vasoactive amines. The relative importance of the direct or indirect effects of C3a on chemoattraction in C3a/GFAP mice during EAE remains to be elucidated.

The outcome of EAE in $C3aR^{-/-}$ and C3a/GFAP mice contrasts sharply with that in $C5aR^{-/-}$ and C5a/GFAP mice. We have previously shown that the onset and progression of EAE in $C5aR^{-/-}$ mice are essentially identical with those in control mice (41). In $C5aR^{-/-}$ mice there was no difference in cellular infiltration, in vitro T cell proliferation, or the expression of proinflammatory cytokines, chemokines, or adhesion molecules. This was particularly surprising because the C5aR is expressed on glial cells, neurons, and infiltrating T cells, and its expression is increased during inflammatory brain conditions, including EAE (13, 42–44). We have also performed EAE in C5a/GFAP mice and observed no difference between transgenic and control mice (R. Reiman et al., manuscript in preparation). For these studies we used four different lines of C5a/GFAP mice and did not find a significant difference in the onset and severity of EAE, cellular infiltration, or production of inflammatory mediators. Our results, using EAE as an autoimmune disease model, contrast sharply with the outcome using a model of acute inflammation in which mice are injected i.p. with LPS. In preliminary experiments C3a/GFAP respond comparably to control mice in terms of survival and serum cytokine levels. In contrast, C5a/GFAP mice are highly sensitive to LPS, with most mice dying within 24–48 h. after treatment, and have marked elevation of proinflammatory cytokines compared with control mice. The differential response of these mice in an autoimmune disease model vs acute inflammation indicates at least some fundamentally distinct roles for the anaphylatoxin receptors and their ligands in the immune response. Furthermore, the classification of C3a and C5a solely as inflammatory mediators reflects a limited knowledge of their inter-relationship with other mediators of immune responses and their temporal and compartmental regulation by carboxypeptidases. In the CNS, C3a/GFAP mice will prove a valuable tool for better understanding of complement anaphylatoxins in acute and chronic models of neurological disease.

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