Complement C5 in Experimental Autoimmune Encephalomyelitis (EAE) Facilitates Remyelination and Prevents Gliosis

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Activation of the classical complement system is known to play a central role in autoimmune demyelination. We have analyzed the role of complement component C5 in experimental autoimmune encephalomyelitis (EAE) using C5-deficient (C5-d) and C5-sufficient (C5-s) mice. Both groups of mice displayed early onset EAE, a short recovery phase, and similar stable chronic courses. However, in contrast to the clinical similarities, marked differences were apparent by histopathology. During acute EAE in C5-d, a delay in inflammatory cell infiltration and tissue damage was observed along with restricted lesion areas, which in C5-s mice were more extensive and diffuse. More striking were the differences in chronic lesions. In C5-d mice, inflammatory demyelination and Wallerian degeneration were followed by axonal depletion and severe gliosis, while in C5-s, the same initial signs were followed by axonal sparing and extensive remyelination. In C5-d, immunohistochemistry and Western blotting showed an increase in glial fibrillary acidic protein and a decrease in neurofilament protein, proteolipid protein, and several pro-inflammatory markers. These results in the EAE model indicate that absence of C5 resulted in fiber loss and extensive scarring, whereas presence of C5-favored axonal survival and more efficient remyelination. (*Am J Pathol* 2003, 163:1069–1080)

The importance of complement as a component of the innate immune system as well as the inflammatory response is well established and its role in the development of inflammatory autoimmune diseases, tissue injury and repair, and disorders affecting the central nervous system (CNS) has been extensively analyzed.^{1–5} Deposition of complement-activation products has been shown in Alzheimer's disease,^{6,7} ischemia/reperfusion injury,⁸ Huntington's and Prion disease,^{9,10} and multiple sclerosis (MS).^{11–15} In the immune-mediated inflammatory demyelinating disease,

multiple sclerosis, and its animal model, experimental autoimmune encephalomyelitis (EAE), myelin, and oligodendrocytes are primary targets of damage.

Studies *in vitro* have demonstrated involvement of the classical complement pathway during demyelination through antibody (Ab)-dependent and Ab-independent mechanisms for complement activation.^{16–18} During demyelination, complement involvement has been demonstrated *in vivo* by inhibition of complement activation or the use of knockout mice. Depletion or inhibition of complement using cobra venom factor or soluble CR1 has been shown to ameliorate EAE in rats.^{19–22} In contrast, complement-fixing anti-myelin oligodendrocyte glycoprotein (MOG) Ab was found to be essential for induction of demyelination in rat EAE induced by MOG.^{22,23} However, MOG-induced EAE in C3 knockout mice revealed conflicting results, with one group reporting lower clinical scores with less inflammation (perhaps indicating protection from demyelination in the absence of C3²⁴), and another, using a higher dose of MOG, showing no differences in clinical score from controls.²⁵ EAE in Factor B knockout mice showed less severe disease than controls, indicating an enhancing role of the alternative pathway.^{24,26} In MOG-induced EAE in C5a receptor (C5aR)-deficient mice, no difference was reported in onset or severity compared to controls.²⁷ Thus, up-regulation of the C5a receptor may not play a key role in EAE.²⁸ In EAE, involvement of components of the membrane attack complex, C5b-9, assembled after cleavage of C5 into C5a, an anaphylatoxin, and C5b, the initiator of assembly with C6–9, are more likely mechanisms. Recently, in studies on Ab-mediated EAE in C6-deficient rats, a reduced level of clinical score and demyelination in the absence of C6 were observed.²⁹

In the present study, we have analyzed the influence of C5 on inflammatory demyelination during the course of

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EAE in C5-deficient (C5-d) and C5-sufficient (C5-s) mice. The results indicate that C5, essential for the formation of C5b-9, is integral for efficient recovery in EAE in that it promotes remyelination, facilitates axonal survival and prevents extensive glial scarring.

Materials and Methods

EAE Induction

Female mice of a congenic outbred strain deficient in C5 (B10.D2/oSnJ; C5-d), and C5-sufficient controls (B10.D2/nSnJ; C5-s), were purchased from Jackson Laboratories (Bar Harbor, ME). Backcrossing of the C5-deficient strain DBA2 with the C5-sufficient strain C57BL/10J established C5 deficiency (7 generations), or C5 sufficiency (17 generations). Mice were routinely investigated at Jackson Laboratories for isoenzyme markers ($n = 10$), as part of the routine checking for genetic integrity. The C5 congenic strains are identical in 23 isoenzymes, H2 and Ea9, but differ in Hc (hemolytic complement, former C5; see <http://jaxmice.jax.org/geneticquality/index.html>). Mice were maintained in a barrier facility according to National Institutes of Health guidelines. EAE was induced by subcutaneous injection of 700 μ g purified guinea pig myelin from frozen spinal cords (Rockland, Gilbertsville, PA), in incomplete Freund's adjuvant containing 70 μ g *Mycobacterium tuberculosis* and H37RA (Difco, Detroit, MI), followed by intravenous injection with 100 ng Pertussis toxin (List, Campbell, CA), on day 0, 2, and 7 post-immunization (p.i.). Clinical signs of EAE were scored daily: 1, tail paralysis; 2, hindlimb weakness and abnormal gait; 3, paraplegia; 4, quadriplegia; 5, moribund or death. From 160 mice injected at 7 to 8 weeks of age, representative animals were perfused with cold PBS for immunohistochemistry/Western blots, or 2.5% glutaraldehyde/Millonig's buffer for histopathology/electron microscopy (EM) at the following disease stages: onset of disease (3 to 4 days after onset, 9 to 14 days p.i.; 23 C5-d, 24 C5-s mice); acute phase (10 to 12 days after onset, 17 to 19 days p.i.; 3 mice each); stabilizing (recovery) phase (21 to 25 days p.i.; 16 C5-d, 23 C5-s); or chronic phase [50 (2 C5-d, 3 C5-s), 90 (2 C5-d, 4 C5-s), 117 and 120 days p.i. (20 C5-d, 18 C5-s)]. In addition, 19 mice died during the course of the experiments.

Hemolytic Activity of Complement and Determination of C5 mRNA

The ability of C5-d and C5-s mouse serum to generate C5b-9 was assessed by hemolytic assay based on the alternative pathway, as described, with minor modification.³⁰ Rabbit erythrocytes, 5×10^5 in 50 μ l dextrose gelatin veronal buffer (DGVB⁺⁺; containing 1% gelatin, 0.15 mmol/L CaCl₂, 1.0 mg MgCl₂, and 2.5% dextrose), were incubated with 10 μ l mouse serum for 60 minutes at 37°C in a microtiter plate in triplicate.³¹ After centrifugation at 1500 rpm for 5 minutes, optical density (OD) of supernatants were measured at 412 nm.

For C5 mRNA, total RNA was isolated from frozen mouse spinal cord and brain. Tissue was homogenized in liquid nitrogen, suspended in lysis buffer containing guanidine isothiocyanate/ β -mercaptoethanol, and centrifuged over a 5.7 mol/L CsCl cushion for 18 hours at 35,000 rpm using a Beckmann rotor, as described previously.³² For reverse transcription-polymerase chain reaction (RT-PCR), 2 μ g of RNA were used for cDNA synthesis, and PCR was carried out at 95°C, 60°C, and 72°C with 35 cycles for 1 minute each, using mouse C5 primers: sense, 5'-ACCCTGCTTCTTCTGGAAAT-3'; anti-sense, 5'-ACCCTGCTTCTTCTGGAAAT-3'. PCR products were separated on a 1% agarose gel. Control C5 mRNA was seen as a 400-bp band.

Histopathology

Slices (1 mm) of glutaraldehyde-perfused cerebrum, cerebellum, and spinal cord (C7, Th2, L2, L5, L6, and S1), were postfixed in 1% osmium tetroxide/Millonig's buffer on ice for 90 minutes, dehydrated in ethyl alcohol (70%, 90%, 95%, and 100%), cleared in propylene oxide and embedded in Epon. One-micron (1- μ m) epoxy sections were stained with toluidine blue for light microscopy (LM) from two or more blocks of tissue from each level. Sections were scored by an investigator blinded to the code, on a scale of 0 to 4 for cell infiltration, de- and remyelination, and Wallerian degeneration, as described.³³ Representative lesions were examined from matching levels of spinal cord from animals from both groups (C5-d and C5-s) with comparable disease courses. For EM, thin sections were cut from representative levels, placed on copper grids, contrasted with uranium and lead salts, carbon-coated, and scanned in a Hitachi H600.

Immunohistochemistry

The following Abs were used for immunohistochemistry (IH): rat Ab to mouse CD4, CD8, interleukin (IL)-2, IL-4, IL-6, IL-10, CD16/32 (Fc III/II receptor), CD19 (B cells), CD54 (ICAM-1), CD62E (E-Selectin), gamma interferon (IFN- γ), tumor necrosis factor- α (TNF- α) (BD PharMingen, San Diego, CA); rat Ab to mouse neutrophils and to antigen F4/80 on macrophages (Serotec, Raleigh, NC); goat Ab to human C1q (Advanced Research Technologies, San Diego, CA); rabbit Ab to rat C9 (Dr. P. Morgan, University of Cardiff, UK), to rat CD59 (Research Diagnostics, Flanders, NJ), to cow glial fibrillary acidic protein (GFAP) for astrocytes (Dako, Carpinteria, CA), to P-Selectin for blood vessels (Dr. S. Green, University of Virginia); and mouse Ab to MAC-1 for macrophages (Roche Diagnostics, Mannheim, Germany) and to neurofilament 200 (NF-200) for intact axons (Sigma, St. Louis, MO). Blocks of PBS-perfused thoracic and lumbar spinal cord embedded in optimal cooling temperature medium (Tissue-Tek, Sakura Finetek, Torrance, CA), were cut as 10- μ m sections. Air-dried sections were fixed in acetone or acetone/methanol (5 minutes each), quenched in 0.3% H₂O₂/PBS (10 minutes) and after blocking for 1 hour in 10% heat-inactivated serum (Vector Laboratories, Burling-

game, CA; DAKO, Carpinteria, CA), incubated with the primary Ab overnight at 4°C, or for control purposes, PBS. Slides were washed 3 × 5 minutes in PBS between incubations. After incubation with a biotinylated secondary Ab (Vector; Jackson ImmunoResearch, West Grove, PA), and avidin/streptavidin complex (Elite kit, Vector), for 1 hour each, positive staining was detected with 3, 3'-diaminobenzidine (DAB kit; KPL, Gaithersburg, MD). Sections were counterstained with hematoxylin, dehydrated, mounted, and scored by a blinded observer on a scale of 0 to 4.

Western Blotting

The same Ab as those used for IH with the addition of: rabbit Ab to VCAM-1 and TNF- α (Santa Cruz Biotechnology Inc., Santa Cruz, CA), mouse Ab to GFAP (Boehringer), and rat Ab to proteolipid protein (PLP) (Dr. A. Gow, Wayne State University, Detroit, MI). Recombinant protein used for controls included TNF α (Santa Cruz or R & D Systems, Minneapolis, MN) and C1q (Advanced Research Technologies). Frozen tissue was homogenized on ice in lysis buffer and protein per lane concentration determined (Bio-Rad DC assay; Bio-Rad, Hercules, CA). 100 μ g of protein were loaded in reducing sodium dodecyl sulfate (SDS) buffer (1:2), separated on 10% or 12% SDS-polyacrylamide gel electrophoresis (PAGE) (Mini-PROTEAN; Bio-Rad), and transferred onto polyvinylidene difluoride membrane for 2 hours (Millipore, Bedford, MA). After blocking in 5% fat-free milk powder/PBS/0.05%Tween 20 (pH 7.4), membranes were incubated in diluted Ab overnight at 4°C. After washing and incubation in horseradish peroxidase-coupled secondary Ab (Pierce, Rockford, IL), immunoreactive bands were visualized by enhanced chemiluminescence (Amersham Biosciences Inc., Piscataway, NJ), on light-sensitive film (Biomax L; Kodak, Rochester, NY). Blots were scanned and quantitated using Ambis Quant Probe software (Ambis Inc., San Diego, CA).

Results

Hemolytic Assay and mRNA Detection Confirms Absence of C5 in C5-d Mice

In B10D2/oSnJ, inbred DBA2, and other mouse strains deficient in C5, genomic DNA studies have revealed a 2-bp deletion in the C5 gene structure.³⁴ This deletion has been reported to cause different forms of C5-deficiency that are cell-specific.^{31,34,35} In C5-d B10D2 mice, C5 protein is absent in liver, which expresses about 20-fold less mRNA.^{31,35} In this study, we evaluated activity of C5 protein in C5-d mice by a hemolytic assay using mouse serum and rabbit erythrocytes (Figure 1A). Rabbit erythrocytes were used since they activate complement by the alternative pathway. Serum from four C5-d and three C5-s mice with acute EAE, or untreated control serum from C5-d and C5-s mice, were examined. With control C5-s serum, a 75-fold higher hemolytic value was observed than with C5-d serum, while during acute EAE, the difference was reduced

Figure 1. Serum hemolytic activity and C5 mRNA expression in C5-d mice. **A:** Mouse serum was tested for the ability of serum complement to lyse rabbit erythrocytes by the alternative pathway. Results are expressed by determining the released hemoglobin at OD_{412 nm}. Control C5-s serum had a hemolytic activity of 2.5 (result not shown). The mean hemolytic activity of five C5-d mice with acute EAE was very low, compared to the mean activity of four C5-s mice. These findings confirmed the lack of activation and consumption of complement during acute EAE in C5-d mice. **B:** RNA obtained from C5-d and C5-s spinal cords and brain from one C5-s mouse was analyzed for C5 mRNA expression by RT-PCR. Analysis of the RT-PCR products on a 1% agarose gel demonstrated only in C5-s mice a band of 400 bp using C5-specific primer.

to about 12-fold. These results confirmed absence of complement hemolytic activity in C5-d serum and provided additional evidence of complement activation *in vivo* in C5-s mice with acute EAE.

We also examined C5 mRNA in C5-d and C5-s mouse CNS tissue by RT-PCR using a pair of mouse C5 primers (Figure 1B). RNA from C5-s spinal cord and brain showed a 400-bp band, as expected for the amplified sequence of mouse C5.³⁴ In C5-d spinal cord, the corresponding mRNA band was not detected.

C5-d and C5-s Mice Develop a Non-Relapsing Course of EAE with Early Onset

100% of C5-d (B10.D2/oSnJ) and C5-s (B10D2/nSnJ) mice sensitized with guinea pig myelin ($n = 160$, Figure 2) developed EAE with early onset (between 6 and 14 days p.i.). In contrast, onset of disease in the inbred C5-deficient parent strain, DBA2, was observed at 18 to

Figure 2. Summary of clinical scores of C5-d and C5-s mice with EAE. The disease pattern of 80 C5-d (B10D2/oSnJ) and 80 C5-s (B10D2/nSnJ) outbred mice is shown as the mean $\pm$ SD. After an initial peak and a recovery period, the animals developed a stable chronic form of EAE, which in C5-s mice was slowly progressive. 75% of C5-d mice and 96% of C5-s mice developed a disease score of 2.5 to 3.5 between days 10 and 11 p.i. Animals were sampled at the onset of disease phase (9 to 14 days p.i.; 23 C5-d, 24 C5-s mice), acute phase (17 to 19 days p.i.; 3 mice each), recovery phase (21 to 25 days p.i.; 16 C5-d, 23 C5-s) or chronic phase [50 (2 C5-d, 3 C5-s), 90 (2 C5-d, 4 C5-s), 117 and 120 days p.i. (20 C5-d, 18 C5-s)].

Table 1. Histopathology of C5-d and C5-s Mice with EAE

Location	Acute EAE (10–12 days p.i.)						Recovery phase (24 days p.i.)						Chronic EAE (120 days p.i.)					
	Cell infiltrates		Demyelination		Remyelination		Cell infiltrates		Demyelination		Remyelination		Cell infiltrates		Demyelination		Remyelination	
	C5-d	C5-s	C5-d	C5-s	C5-d	C5-s	C5-d	C5-s	C5-d	C5-s	C5-d	C5-s	C5-d	C5-s	C5-d	C5-s	C5-d	C5-s
Upper spinal cord	1.67	2	0.75	1	0	0	1.17	1.56	1.17	1.89	0	0.67	0.5	0.58	0.33	0.58	0.08	0.5
Lower spinal cord	2	2	0.58	2	0	0	2	1.67	1.47	1.04	1.06	1.17	0.58	0.33	0.83	0.5	0.58	1

Distribution and severity of histopathologic signs. Toluidine blue-stained epoxy sections of representative mice were evaluated with 0.5 (+/–), 1 (+), 2 (++), 3 (+++), and 4 (++++ for inflammatory cell infiltrates, demyelinated and remyelinated axons. Means were calculated from values from up to 4 slides of each level of brain and spinal cord. Upper spinal cord represents C7, Th2, and L2 levels, lower spinal cord L5, L6, and S1 levels. During acute EAE (3 days post-onset), demyelination was less severe in C5-d than C5-s mice. Remyelination was more prominent in the recovery (24 days p.i.) than in the chronic phase (120 days p.i.) of EAE in C5-s mice, while C5-d mice developed intense gliosis. A trend towards a rostral shift in lesion activity from the lower spinal cord towards the brain could be observed with time.

24 days (not shown). Clinical signs up to grade 3.5 were detected in 142 of 160 mice between 6 and 10 days p.i. and in the remainder between 11 and 14 days. The disease pattern of most C5-d and C5-s B10.D2 mice involved an acute phase, a short recovery (stabilizing) phase, and a stable chronic phase. The latter was essentially identical in both groups except that in C5-s, the course was slightly more progressive (Figure 2). High standard deviations in clinical outcome were observed in each group, probably a reflection of the high numbers studied ($n = 80$ for each group), and the high genetic variability associated with outbred strains. Mice injected with complete Freund's adjuvant (CFA) and Pertussis developed no signs of EAE.

Presence of C5 Influences CNS Lesion Extent, Tissue Destruction, and Repair

Despite the similarity in clinical outcome between the two groups (Figure 2), and a great similarity in lesion distribution at the level of histopathology, there were marked differences. Lesions in C5-d mice in the early acute phase (10 to 12 days p.i., 3 days after onset), were clearly demarcated and restricted in extent with less inflammation (Table 1), and demyelination (Figure 3, A and B). In contrast, lesions in C5-s mice were widespread and diffuse, with extensive inflammation, demyelination, and Wallerian degeneration (Figure 3, C and D). Inflammation and demyelination were more pronounced in the lower spinal cord of C5-d and C5-s mice, with C5-s displaying more advanced disease (Table 1). At the height of acute EAE (17 days p.i., 7 days after onset), lesion activity remained prominent in the lower spinal cord but extended rostrally in both mice (data not shown). Lesions in C5-d (B10D2/oSnJ and DBA2) mice contained more Wallerian degeneration, while inflammation and demyelination of DBA2 mice was similar to C5-s mice (data not shown).

During the recovery phase (21 to 24 days p.i., 17 days after onset), inflammation and demyelination were exten-

sive in lesions of C5-d mice, especially in the lower spinal cord (Figure 3, E and F; Table 1). Wallerian degeneration was present along the margin of the confined lesions (Figure 3E), and some remyelination was apparent. In contrast, C5-s mice revealed remarkable and widespread early remyelination, although inflammation, demyelination, and Wallerian degeneration were still present but less pronounced. (Figure 3, G and H; Table 1).

In chronic C5-d mice (50 days p.i.), lesions showed increased gliosis, Wallerian degeneration, and reduced inflammation and demyelination (mainly in brain and cervical regions; data not shown), which developed during the later chronic periods (90 and 120 days p.i.), into a subpial glial scar (Figure 3, I and J; Table 1). In contrast, C5-s mice with chronic EAE showed abundant remyelination in subpial regions of the spinal cord, where less inflammation and demyelination occurred within mildly gliotic areas (Figure 3, K and L; Table 1).

Thus, inflammation, demyelination, and Wallerian degeneration were more widespread and appeared earlier in C5-s mice, whereas lesions in C5-d were confined to narrow zones displaying features similar to C5-s lesions, with destruction continuing into the chronic phase. In C5-d mice, gliosis and fiber loss were the main features, while axonal preservation and remyelination prevailed in C5-s mice.

Astroglial Gliosis and Nerve Fiber Damage in C5-d Lesions

The distribution of gliosis and glial scar formation was further evaluated by IH and Western blot analysis of GFAP, PLP and NF-200. The subpial margins of spinal cord stained more intensely for GFAP in C5-d than C5-s mice during acute and chronic EAE (data not shown), as expected from the increase in reactive astrocytes in chronic lesions (see Figure 3, I and J). Western blots confirmed increased levels of total GFAP protein in spinal cord homogenates of C5-d mice during chronic EAE (Figure 5A). The markedly-reduced PLP level during

Figure 3. Histopathology of acute, recovery, and chronic phases of EAE in C5-d and C5-s mice. Toluidine-blue-stained epoxy sections (1 μ m) from the upper lumbar of C5-d mice with acute EAE (**A** and **B**), revealed well-demarcated, confined lesions with inflammation and a narrow rim of demyelinated axons. Lesions of C5-s mice with acute EAE (**C** and **D**) are more diffuse and display inflammation and demyelination in addition to Wallerian degeneration (**D**, lower left), dystrophic axons (**D**, center right), and myelin ovoids (**D**, left). In the recovery phase of EAE, C5-d mice (**E** and **F**, cervical spinal cord) show extensive Wallerian degeneration (dense droplets, lower right) and demyelination. In contrast, extensive remyelination was present in C5-s mice (**G** and **H**, S1 spinal cord), shown by numerous thinly myelinated fibers along the meningeal surface (**H**). Numerous fibrous astrocytes with pale nuclei occur within the remyelinated zone. During chronic EAE, C5-d mice (**I** and **J**) developed a prominent zone of intense gliosis at the margin of the spinal cord, from which nerve fibers and oligodendrocytes were depleted. Lesions in C5-s mice (**K** and **L**) displayed numerous remyelinated axons at the edge of the spinal cord, where some gliosis is also present.

Figure 4. Immunohistochemistry (IH) of C5-d and C5-s mice with EAE. Neurofilament (NF-200): Sections from C5-d with chronic EAE revealed weaker and scattered staining of NF-200 (**A**) in areas corresponding to a chronic lesion, compared to the robust, densely-packed staining in sections of C5-s mice (**B**), where most axons survived. P-Selectin: In chronic EAE, staining for the endothelial adhesion molecule, P-Selectin, was lower in C5-d (**C**) than C5-s (**D**), perhaps indicating reduced infiltration of inflammatory cells into the tissue. MAC-1: Increased staining of macrophages in C5-s mice (**E**), during acute disease suggested enhanced phagocytosis of myelin debris in C5-s, in comparison to C5-d mice (**F**). IL-6: In acute EAE, IL-6 was enhanced in reactive astrocytes in C5-d (**G**), in comparison to C5-s mice (**H**). Magnifications: **A** and **B**, $\times 63$; **E**, **F**, **G**, **H**, $\times 625$.

acute EAE in both groups of mice (Figure 5B) was significantly up-regulated during chronic EAE in C5-s, but not C5-d mice. The reduced level of NF-200 staining observed in lesions of C5-d mice with chronic EAE (120 days p.i.; Figure 4A) indicated nerve fiber damage and contrasted with higher levels of NF-200 in remyelinated lesions of C5-s mice (Figure 4B). The finding of axonal depletion in C5-d mice correlated well with observed glial scarring and lack of axons (Figure 3, J and L).

In summary, increased GFAP and reduced NF-200 expression in the CNS of C5-d mice with chronic EAE

confirmed marked gliosis seen by LM in C5-d mice, whereas up-regulated NF-200 correlated with axonal sparing and remyelination noted in chronic C5-s mice.

Ultrastructural Differences Confirm an Effect of C5 on Lesion Formation

EM of spinal cord (Figure 6) from representative C5-d mice and controls confirmed differences seen by LM (Figure 3). During the acute phase of EAE, typical signs

Figure 5. Protein expression by Western blot analysis. 10 μ g (GFAP, PLP, and C1q) or 100 μ g (VCAM-1 and TNF- α) of spinal cord homogenate separated on 10% to 12% SDS-PAGE and transferred by Western blotting were probed with specific antibodies for various cytokines, adhesion molecules and myelin proteins. Results performed in triplicate (except for PLP, VCAM-1) are expressed as relative values of densitometric numbers with the value of the band obtained for untreated C5-s spinal cord taken as 1.0. GFAP: GFAP (A) up-regulated by IH during the chronic phase in C5-d in comparison to C5-s mice was confirmed, indicating enhanced astrogliosis in C5-d. PLP: The myelin protein, PLP (B), was shown to be down-regulated during the acute phase in both groups, indicating myelin breakdown. In chronic C5-s mice, almost baseline levels of PLP were detected, suggesting remyelination. C1q: In C5-d mice, the complement protein, C1q (C), was decreased in comparison to C5-s during acute and even more so during chronic EAE, indicating an increased activation of complement in C5-s mice. VCAM-1: A robust inflammatory response, indicated by up-regulation of the adhesion molecule, VCAM-1 (D), during the acute phase, was sustained further in C5-d mice during chronic disease, reaching levels similar to levels in chronic C5-s. TNF- α : Up-regulation of TNF- α was more prominent in C5-d mice than C5-s during acute EAE, whereas levels dropped in C5-d mice but not C5-s mice during chronic EAE ($P < 0.026$) (E).

Figure 6. Differences between chronic lesions in C5-d and C5-s mice by EM. An area of a spinal cord from a C5-d mouse (**A**, 24 days p.i.) shows destructive lesions with ongoing Wallerian degeneration and a few inflammatory cells, whereas in C5-s (**B**, 24 days p.i.) widespread early remyelination is clearly visible. During chronic EAE (**C**, 120 days p.i.), differences were equally obvious, with fibrous astrogliosis predominating in C5-d mice, and advanced remyelination and a less gliotic parenchyma in matching sections of C5-s mice (**D**). Magnifications: **A**, ×3400; **B**, ×4600; **C**, ×5400; **D**, ×6000.

Table 2. Immunocytochemistry of C5-d and C5-s Mice with EAE

Marker	Acute EAE		Chronic EAE	
	C5-deficient	C5-sufficient	C5-deficient	C5-sufficient
CD4	++	++	+	+/-
CD8	-	-	+/-	-
CD19	-	+/-	+/-	+
Macrophage	++	++	+/-	+
Neutrophil	++	+++	-	+/-
IL-2	+	++	+	+
IL-4	+	+/-	+	++
IL-6	+++	++	+	+
IL-10	++	++	++	+/-
IFN- γ	+/-	+	+/-	+
TNF- α	+	++	+	++

Summary of inflammatory markers investigated by IH. In acute EAE, immunostaining indicated extensive expression of IL-6 in C5-d mice in inflammatory cells as well as in astrocytes, whereas in C5-s mice, higher expression of neutrophils, TNF α , IL-2 and B cells (CD19), are apparent. In chronic EAE, a few CD8 T cells in C5-d mice suggest an environment of ongoing degeneration, while in C5-s mice the enhanced presence of macrophages and B cells may be consistent with remyelination. In general, the presence of CD4 T cells, macrophages and neutrophils were typical in spinal cord lesions from animals with acute EAE, whereas B cells were more a feature of the chronic phase of the disease. In the acute phase, note the predominant involvement of TH1 cells (IL-2) in C5-s mice, while in the chronic phase, a shift to the TH2 type (IL-4) was seen.

like edema, fibrin deposits, polymorphonuclear and mononuclear cell infiltrates, and demyelinated and dystrophic axons were observed in lesions of both groups of mice (data not shown). Lesions from the recovery phase (21 days p.i., 17 days after onset) for C5-d mice characteristically contained perivascular cuffs, Wallerian degeneration, and fibrous astrogliosis, indicating an inflammatory degenerative process, while a few healthy oligodendrocytes were still present. In contrast, lesions in C5-s mice revealed, along the subpial margin of the spinal cord, an abundance of inflammatory cells, particularly neutrophils undergoing apoptosis (not shown), myelin debris-laden macrophages, fibrin, collagen, and plasma cells. Apoptotic infiltrating cells were not prominent in C5-d mice with EAE. In the recovery phase, ongoing nerve fiber degradation was seen in C5-d lesions, while repair processes were well advanced in C5-s mice, in which early CNS remyelination was apparent over large areas at the edge of the spinal cord (Figure 6, A and B). Remyelination of CNS axons by invading Schwann cells was occasionally observed. During chronic EAE (50 to 120 days p.i.), lesions in C5-d mice developed the appearance of gliotic scars in subpial zones (Figure 6C). At the junction of the glial scar and surrounding white matter, a few oligodendrocytes and scattered remyelinated axons were detected. In contrast, in C5-s mice, CNS remyelination by oligodendrocytes (Figure 6D) and peripheral nervous system (PNS) myelination by Schwann cells were prominent along with gliosis and some apoptotic cells.

In summary, ultrastructural analysis confirmed that CNS lesions in C5-d mice were progressively destructive and led to axonal loss and fibrous astrogliosis in the chronic phase, while lesions in C5-s were reversible to a large extent, displaying axonal preservation and varying degrees of remyelination.

Adhesion Molecules, Inflammatory Markers, and Complement in Lesion Formation

Since complement C5a and MAC regulate adhesion molecule expression,³⁶⁻³⁸ and MAC is involved in inflamma-

tory demyelination and increases P-Selectin in EAE, which is also involved in the recruitment of inflammatory cells into the CNS in EAE,^{39,40} we investigated expression of E- and P-Selectin, VCAM-1, complement receptor CR1, and CD11a ligand, ICAM-1 (CD54). CD54 and the receptor CR1 as well as P-Selectin and MAC-1 (CR3) were expressed in acute EAE to a lower extent in C5-d than C5-s mice (Figure 4, C to F; Table 2). Increased P-Selectin was confirmed by Western blotting (data not shown). Expression of VCAM-1 (Figure 5D) and ICAM-1 (not shown) increased in C5-s during acute EAE more than in C5-d.

Expression of the complement protein, C1q, was low in naïve mice from both groups, but increased in both groups during acute EAE and in C5-s during chronic EAE (not shown). Staining for C3d, C9 and CD59 was detected at comparable levels in both groups in acute EAE (data not shown).

Neutrophils were more common in C5-s than C5-d in acute EAE. C5-d mice with chronic EAE showed fewer macrophages and absence of neutrophils (Table 2). CD4 cells were similarly present in both groups in acute EAE and were reduced in C5-s mice with chronic EAE. Expression of IL-2 and IFN- γ , markers for TH1-type cells, was lower in C5-d mice. In contrast, IL-10, a TH2-type cytokine, was expressed at high levels during the acute phase in both groups, whereas IL-4, another TH2-type cytokine, was elevated in C5-d over C5-s during acute EAE. Levels of IL-4 rose further in C5-s mice but remained stable in C5-d mice in the chronic phase.

Among the cytokines examined by IH and semiquantitatively by Western blotting, we found the following changes: IL-6, known to be expressed by both inflammatory and resident CNS cells, occurred at higher levels by astrocytes in C5-d mice during acute EAE (Figure 4G), whereas C5-s displayed lower-level expression (Figure 4H; Table 2). During chronic EAE, lower levels of IL-6 were expressed on the cell surface whereas Western blots confirmed elevated levels of IL-6 in C5-d over C5-s mice (data not shown). TNF α , also expressed by both

infiltrating and resident cells, was expressed at higher levels in astrocytes in C5-s than C5-d mice during acute EAE. Western blots revealed nearly identical expression of TNF α during acute EAE and a statistically significant decrease during the chronic phase in C5-d mice ($P < 0.026$), while levels in C5-s were markedly decreased in the acute phase and elevated in the chronic phase (Figure 5E).

During the acute phase, CD19⁺ B cells were not detectable in C5-d and only a few were present in C5-s mice. However, during the chronic phase, C5-s displayed an increase in the B-cell population (Table 2). Differences in phagocytosis of myelin debris between the two groups were observed, which may explain the presence of gliosis *versus* remyelination during chronic EAE. Recruitment of macrophages, shown by immunostaining with antibodies F4/80 and MAC-1, and expression of FcII/III (CD16/32) receptor, were slightly elevated in C5-s mice during the acute phase when inflammation was more prominent (Figure 4, E and F). In chronic EAE, macrophage markers and Fc receptors remained higher in C5-s mice.

Discussion

Among the basic tenets of inflammation is that it is a protective mechanism in which an early pro-inflammatory response leads to cell death and tissue destruction and that this gives way to an anti-inflammatory phase, which down-regulates the response and prepares the tissue for repair, thus achieving homeostasis. To date, very little is known about factors participating in CNS tissue repair after demyelination. With the present EAE paradigm in C5-d and C5-s mice, it is precisely this return to tissue homeostasis which appears to be disrupted in mice with C5 deficiency, a feature characterized structurally by disruptions in axonal preservation and remyelination.

Along these lines, of the numerous differences in disease response between C5-d and C5-s mice described herein, those in long-term affected animals strikingly underscored the importance of C5 in the ability of the CNS to remyelinate after inflammation-mediated damage. In this regard, C5-d mice with chronic EAE displayed lesions intensely gliotic and depleted of axons, while C5-s showed remyelination, sometimes to near normal levels. These findings are also supported by IH and semiquantitative Western blots in C5-d mice showing up-regulation of GFAP and decreased expression of NF-200, and in C5-s mice with chronic EAE showing abundant NF-200-positive fibers at sites of remyelination, together with an increase in myelin protein, PLP. By extending the studies well into the chronic phase of EAE and by examining long-term manifestations of the C5 defect, this model identified C5 as an essential factor in the repair of affected white matter through its role in axonal preservation. C5a, a potent inflammatory mediator, is also a pleiotropic cell activator and is neuroprotective *in vitro*.^{41–43} However, C5aR-deficient mice have been shown to develop EAE with similar clinical scores and lesion activity to control mice,²⁶ but since animals were only studied to

day 20 p.i. and nerve fiber pathology was not examined, it is difficult to compare the findings to those of the present study, where C5-d and C5-s mice also displayed similar scores but markedly different nerve fiber pathology over a much longer time period. These results underline the need for careful histopathological examination even when clinical scores are similar. Since C5b-9 can also generate inflammatory mediators such as leukotrienes and prostaglandins,^{44,45} and is directly implicated in myelin damage and demyelination,³ both C5a and C5b-9 in C5-s mice may have contributed to the more widespread and invasive inflammatory response and increase in neutrophils in acute EAE, as described.^{41,46} Our findings are consistent with reports on a critical role for C5b-9 in C6-deficient rats with EAE, where its expression was associated with demyelination and axonal damage.^{29,39} In addition, the more severe axonal damage seen here in C5-d mice with chronic EAE also indicates an absence of the reparatory role of C5b-9 and not C5a.

Involvement of complement in the inflammatory response also includes an up-regulation of endothelial adhesion molecules. Our data, showing an increase in expression of P-Selectin and MAC-1 in C5-s mice, are consistent with the ability of C5a and C5b-9 to increase adhesion molecule expression^{36,38} and confirm an earlier finding of increased P-Selectin in C6-deficient rats with EAE.³⁹ It is also likely that a number of cytokines and chemokines (essential regulators of adhesion molecule expression) are also critical in this function.

When parameters involved in the immune response, such as cell types, cytokines, and complement were compared, differences were found between C5-d and C5-s mice. An increased neutrophil population was seen in lesions in C5-s mice with acute EAE (10 days p.i.; Figure 3, A and B), many of them undergoing apoptosis, and there was no persistence of inflammatory cells into the chronic phase. CD8 cells were slightly increased in C5-d mice with chronic EAE and this is also consistent with prolonged cytotoxicity and axonal damage. We found small differences in myelin phagocytosis, but no association of demyelination with neutrophils, and phagocytosis of apoptotic lymphocytes, features also described in other murine models.^{47,48} In C5-s, increased IL-2 in the acute phase and higher levels of IL-4 and TNF- α in chronic EAE may also correlate with lesion recovery and the proposed role of TNF- α in remyelination.⁴⁸ The increased IL-6 in astrocytes, shown by IH and semiquantitative Western blotting in C5-d mice with acute EAE, supports astrocytic reactivity. Enhanced IL-6 levels are known to be associated with increased polymorphonuclear cell apoptosis (seen in C5-s mice with acute EAE), and when they remain elevated into the chronic phase (as in C5-d mice) such levels of IL-6 may be detrimental to the tissues, as has been described in other chronic inflammatory diseases.⁴⁹

Taken together, our findings implicate the importance of C5 in the establishment of an extensive and invasive early inflammatory lesion in EAE and in causing more severe demyelination. Paradoxically, the more detrimental response in the presence of C5 was followed by more extensive axonal preservation and vigorous remyelina-

tion, as seen during the recovery phase and chronic EAE. Precisely what mechanism underlies the above effects remains to be shown but the phenomenon is unique to EAE. The role of C5 deficiency has also been examined in models of neurodegeneration,⁵⁰ in which the same congenic pair of C5-s and C5-d mice (B10.D2/SnJ) were applied to the study of excitotoxic damage due to kainic acid. C5-d mice had more neuronal death and greater induction of astrocyte mRNAs (GFAP, apoE, and apoJ). In the same work, lipopolysaccharide induced up-regulation of IL-6 and TNF- α in C5-d. These enhanced responses suggest that C5 deficiency modifies responses of neurons and astrocytes to neurodegenerative stimuli. Our data are consistent with these findings and suggest that C5 deficiency may modify the response of glial cells, eg, oligodendrocytes, to injury.

The efficiency of C5b-9 assembly is regulated by inhibitory proteins such as CD55 and CD59 in a species-restricted manner.⁴⁰ Oligodendrocytes express both CD55 and CD59 and are resistant to complement attack.^{51,52} In the absence of these inhibitors, these cells are 3- to 5-fold more susceptible to MAC-induced cell death.^{53,54} A role for MAC as an effector of myelin destruction has been studied *in vitro*, as shown by its requirement for demyelination in CNS explant cultures.¹⁶ This is thought to be caused by myelin-inserted MAC channels, which induce hydrolysis of myelin basic protein via activation of Ca²⁺-dependent neutral proteases.^{55,56} Myelin is susceptible to MAC-induced damage, since CD55 is not expressed on myelin.⁵⁶ In addition, oligodendrocytes,⁵⁷ like other cells,^{58,59} are able to eliminate potentially lethal MAC from the cell membrane and its presence is known to increase survival of oligodendrocytes *in vitro*, by rescuing the cells from apoptotic death induced by growth factor deprivation or TNF- α .^{60,61} More pertinent to the present study on C5 deficiency and EAE are preliminary results from these laboratories,⁶² demonstrating that C5 deficiency can lead to incomplete clearance of apoptotic oligodendrocytes, thereby possibly negatively affecting the repair process. These conclusions are consistent with a dual role of MAC, one in damaging the myelin membrane and the second in enhancing survival of oligodendrocytes. Our findings thus contribute to the understanding of the evolution of the inflammatory demyelinating lesion in MS and EAE, in which complement plays an important role.

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