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Effects of Complement C5 on Apoptosis in Experimental Autoimmune Encephalomyelitis[1]

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Complement activation is involved in the initiation of Ab-mediated inflammatory demyelination in experimental autoimmune encephalomyelitis (EAE). At a sublytic dose, the C5b-9 membrane attack complex protects oligodendrocytes (OLG) from apoptosis. Using C5-deficient (C5-d) mice, we previously showed a dual role for C5: enhancement of inflammatory demyelination in acute EAE, and promotion of remyelination during recovery. In this study, we investigated the role of C5 in apoptosis in myelin-induced EAE. In acute EAE, C5-d and C5-sufficient (C5-s) mice had similar numbers of total apoptotic cells, whereas C5-s had significantly fewer than C5-d during recovery. In addition, although both groups of mice displayed TUNEL$^+$ OLG, there were significantly fewer in C5-s than in C5-d during both acute EAE and recovery. Gene array and immunostaining of apoptosis-related genes showed that Fas ligand expression was higher in C5-s. In C5-s mice, Fas$^+$ cells were also higher than in C5-d mice in acute EAE; however, these cells were significantly reduced during recovery. Together, these findings are consistent with the role of C5, possibly by forming the membrane attack complex, in limiting OLG apoptosis in EAE, thus promoting remyelination during recovery. ***The Journal of Immunology*****, 2004, 172: 5702–5706.**

Complement has a well-established role in the development of inflammatory and autoimmune diseases of the CNS (1). In multiple sclerosis (MS)[3] and its animal model, experimental autoimmune encephalomyelitis (EAE), myelin and oligodendrocytes (OLG) are primary targets of cellular and humoral immune effectors (2). OLG are depleted in lesions of MS and EAE (3, 4), and this is thought to be mediated, in part, by apoptosis (5–8). This apoptosis is brought about through the interaction of TNFα and Fas ligand (FasL) with their respective receptors (5, 8). Myelin loss that is sustained from the acute phase of the disease to the chronic stage may occur due to a failure in remyelination secondary to OLG death and an inability of OLG precursors to expand (9).

The membrane attack complex (MAC) of complement, C5b-9, was also involved in MS and EAE, as shown by the presence of C5b-9 neoantigens in the spinal fluid of MS patients (10), on the surface of damaged myelin and OLG, and in degraded myelin within macrophages (11). Assembly of MAC begins when C5 is cleaved by C5 convertases, generating C5a and C5b. C5a participates in inflammation as anaphylatoxin and as a chemotactic factor, and C5b initiates MAC formation by reacting with C6–C9 (12). MAC assembly is down-regulated by inhibitory proteins, such as CD55 and CD59, in a species-restricted manner. MAC, but not C5b-7 or C5b-8, is required to demyelinate CNS explant cultures (13), and this is believed to be caused by the MAC-induced hydrolysis of myelin basic protein through the activation of Ca^{2+}-dependent neutral proteases (14, 15). Because it lacks CD55, myelin is susceptible to complement attack (16). In contrast, OLG are more resistant to MAC, because they express both CD55 and CD59 (17). OLG are able to eliminate potentially lethal MAC from the membrane (18, 19). At a sublethal dose, MAC increases the survival of OLG in vitro by inhibiting the mitochondrial pathway of apoptosis induced by growth factor deprivation or TNFα (20, 21). This protection by MAC is, at least in part, through posttranslational regulation of BAD (21).

The possible role of MAC in immune-mediated demyelination was evaluated in an EAE model using C6-deficient rats (22, 23). In these studies, demyelination and disease activity were significantly increased in the presence of MAC. Recently, we investigated the role of the terminal complement cascade in EAE using C5-deficient (C5-d) mice (24). As expected, more pronounced inflammation and demyelination were seen in C5-sufficient (C5-s) mice compared with the C5-d mice in acute EAE. However, the most striking difference was found in chronic EAE, in which the lesion was extensively remyelinated in C5-s mice, whereas severe gliosis with axonal loss were seen in C5-d (24). These findings are consistent with the in vitro effect of MAC to increase the survival of OLG, because C5a does not play a role in acute EAE (25) or in OLG survival (20). Therefore, we investigated the role of C5 and the possible involvement of terminal complement pathway on apoptosis in EAE using C5-d mice.

In this study, we show that total apoptotic cells, similarly high in both groups of mice in acute EAE, had decreased during remission in C5-s mice, but not in C5-d. Apoptotic OLG were greater in number in C5-d than in C5-s mice during the acute and recovery phases of EAE. The expression of FasL and Fas were higher in acute EAE,

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[3] Abbreviations used in this paper: MS, multiple sclerosis; EAE, experimental autoimmune encephalomyelitis; OLG, oligodendrocyte; FasL, Fas ligand; MAC, membrane attack complex; C5-d, C5 deficient; C5-s, C5 sufficient; C5b-9, terminal complement complexes consisting of C5b, C6, C7, C8, and C9 proteins.

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followed by a decrease during remission in C5-s mice, whereas FasL and Fas$^+$ cells remained high in C5-d mice in remission. These data suggest that C5 may be required for clearance of apoptotic inflammatory cells and protection of OLG from Fas-dependent apoptosis, possibly through activation of the terminal C5b-9 complex.

Materials and Methods

Induction of EAE

Adult female mice of a congenic outbred strain deficient in C5 (D10.D2/0SnJ) and C5-s controls (B10.D2/nSnJ) were generated by backcrossing C5-d for 7 generations and C5-s for 17 generations (The Jackson Laboratory, Bar Harbor, ME). Mice were maintained in a barrier facility according to National Institutes of Health guidelines. To induce chronic relapsing EAE (24), mice were immunized at 7–8 wk of age with purified guinea pig myelin in an equal volume of IFA containing 70 μg of *Mycobacterium tuberculosis* H37RA (Difco, Detroit, MI), and 100 ng of pertussis toxin (List Biologicals, Campbell, CA) given i.v. on the same day. Mice were weighed and observed daily for clinical signs of EAE and graded by a blinded individual for neurological deficits on a scale of 0–5 as follows: 0.5, tail weakness; 1.0, tail paralysis; 2.0, hindlimb weakness and abnormal gait; 3.0, paraplegia; 3.5, tetraplegia; 4.0, quadriplegia; and 5.0, moribund state or death. Mice, under terminal anesthesia, were sacrificed 10–12 days and 23–24 days postimmunization (acute EAE and recovery phase, respectively) by transcardial perfusion of cold 4% paraformaldehyde in PBS for immunocytochemistry and cold sucrose in PBS for RNA isolation.

Apoptosis detection by TUNEL assay and double labeling

For TUNEL staining, frozen (3–5 μm) or paraffin-embedded sections of cervical spinal cords were used. Cryostat sections were air-dried and fixed in 1% paraformaldehyde in 0.1 M PBS (pH 7.4) for 10 min. Endogenous peroxidase was quenched with 0.03% hydrogen peroxide in PBS for 5 min, as described (26). Paraffin sections were first deparaffinized, and then endogenous peroxidase was quenched with 3% hydrogen peroxide for 5 min at room temperature. To detect DNA strand breaks, TdT-dependent incorporation of dUTP was measured using ApopTag In Situ Apoptosis Detection (Intergen, Purchase, NY), as described (26). Briefly, sections were incubated for 1 h at 37°C with TdT enzyme, washed, incubated with anti-digoxigenin peroxidase-conjugated Ab, and then developed using ImmunoPure metal enhanced diaminobenzidine tetrachloride substrate (Pierce, Rockford, IL). Apoptotic cells were identified as TUNEL-positive cells showing either nuclear karyorhexis or pyknosis. Apoptosis was assessed by examining more than three sections per animal, and expressed as an average percentage of total TUNEL$^+$ cells per section.

For double labeling, cryostat sections were first stained for TUNEL, and then stained with mouse monoclonal anti-OLG/myelin Ab (MAB 328; Chemicon, Temecula, CA) using the Vector MOM Immunodetection kit (Vector Laboratories, Burlingame, CA). After blocking 1 h with the IgG blocking reagent, sections were incubated overnight at 4°C with MAB 328 (1:10,000) and then with biotin-labeled anti-mouse IgG. Sections were washed, incubated with ABC (avidin/biotin complex) Elite complex, and then developed using Vector NovaRED as a substrate (Vector Laboratories). Slides were washed, dehydrated, cleared in xylene, and permanently mounted. By counting MAB-positive cells with visible nuclei, the total number of TUNEL-positive OLG was determined by examining three different sections per mouse using a ×40 objective. TUNEL assay and immunostaining were independently evaluated by two blinded investigators. We made a mean for the three spinal cord sections used per mouse. This mean was then used to calculate the average + SEM per time point. We used Student's *t* test to determine the statistical significance of various data.

Immunohistochemical detection of FasL and Fas proteins

After quenching with hydrogen peroxide, slides were washed for 5 min in 0.15 M PBS (pH 7.4) and then blocked 1 h in PBS containing 10% goat serum (DAKO, Carpinteria, CA). After washing, sections were first incubated overnight at 4°C with a 1/200 dilution of rabbit anti-Fas (sc-7886; Santa Cruz Biotechnology, Santa Cruz, CA) or anti-FasL IgG (sc-4240; Santa Cruz Biotechnology), and then with goat anti-rabbit HRP-conjugated IgG (Jackson ImmunoResearch Laboratories, West Grove, PA). Specific reaction was developed using NovaRED (Vector Laboratories). Nuclei were counterstained with Harris's hematoxylin (Sigma-Aldrich, St. Louis, MO). Control sections were prepared by staining without the primary Ab, staining with control isotype IgG, and also by preincubating the first primary Ab with a blocking peptide (Santa Cruz Biotechnology).

Some sections were double stained to identify Fas$^+$ OLG. Cryosections were stained for Fas, as described above, and were further incubated overnight at 4°C with MAB 328 (1:10,000) in 0.1% Tween PBS. Slides were washed several times in PBS, reacted with anti-mouse biotinylated secondary Ab (1:250) in PBS, and then with avidin-biotin-peroxidase complex (MOM kit, Vector Laboratories). After washing, sections were reacted with NovaRED. Fas$^+$ OLG in spinal cord sections were quantified by light microscopy using a ×40 objective.

Isolation of total RNA and gene array analysis

Total RNA was extracted from cervical spinal cords using a guanidine isothiocyanate method and by ultracentrifugation on a 5.7 M CsCl2 cushion for 18 h at 35,000 rpm using a SW 60 Beckman rotor as previously described (26). RNA was further purified using an RNeasy column (Qiagen, Santa Clarita, CA). Spinal cords were pooled from three C5-d and three C5-s mice during acute EAE. Gene array analysis was performed using the mouse apoptosis GEArray Q series kit (SuperArray, Bethesda, MD) consisting of 96 apoptosis-related genes and 4 housekeeping genes printed on a nylon membrane. Total RNA (2 μg) was used as a template for [α-^{32}P]dCTP cDNA probe synthesis, according to the manufacturer's protocol. Membranes were hybridized with labeled probe, washed, and exposed to Phosphor screen (Amersham Biosciences, Piscataway, NJ). Radiographic densities were scanned, and the integrated density volume was calculated using Imagequant 5.0 software (Amersham Biosciences, Piscataway, NJ) and expressed as density ratios to β-actin. RT-PCR amplification was performed using 2 μg of total RNA for the first-strand synthesis followed by amplification in the presence of specific primers for FasL (5′-AGCTACCTGGGGGACGTATT-3′ and 5′-TTCTCTGTGCCTCTG CATTG-3′) and β-actin (5′-ATCTGGCACCACACCTTCTACAAT GACTGCG-3′ and 5′-CGTCATACTCCTGCTTGCTGATCCACATCT 3′). The amplification consisted of 35 cycles of denaturation for 1 min at 94°C, annealing for 1 min at 60°C, and extension for 1 min at 72°C.

Results

Apoptotic cells in acute EAE and recovery

As previously reported, all C5-s and C5-d mice developed EAE, and the disease pattern of most mice involved an acute phase, a short recovery phase, and a stable chronic phase (24). Clinical signs up to grade 3.5 were detected between day 10–12 postimmunization. By TUNEL staining and nuclear morphology, apoptotic cells were assessed during acute EAE (days 10–12 postimmunization) and during recovery (days 23–25 postimmunization). In acute EAE, the level of TUNEL$^+$ apoptotic cells in C5-s (372 ± 89) was similar to that of C5-d (362 ± 90) mice (Fig. 1). During recovery, the number of apoptotic cells remained high in C5-d mice (395 ± 15), but decreased significantly in C5-s mice (255 ± 48; $p < 0.04$). In the acute phase, the majority of TUNEL$^+$ cells were inflammatory cells. They consisted of lymphocytes and monocytes and were located within the inflammatory infiltrates involving the posterior and anterior funiculi (Fig. 2*B*). Apoptotic cells were also seen in white matter, and some were identified as OLG by double staining (Fig. 2*D*).

Apoptotic OLG in acute EAE and recovery

We counted apoptotic OLG by reacting the TUNEL-stained sections with MAB 328, an Ab specific for OLG and myelin (Figs. 2*D*

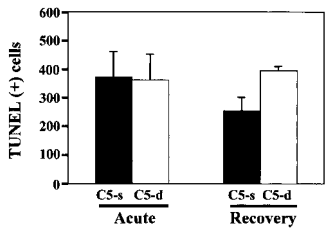

FIGURE 1. Apoptosis in C5-s and C5-d mice with EAE. Quantitative evaluation of apoptosis was performed on cervical spinal cord sections in C5-s ($n = 5$) and C5-d ($n = 5$) mice and expressed as mean ± SEM using paraffin sections and TUNEL assay. Compared with C5-s mice, C5-d mice showed statistically significant higher numbers of apoptotic cells during recovery ($p < 0.04$).

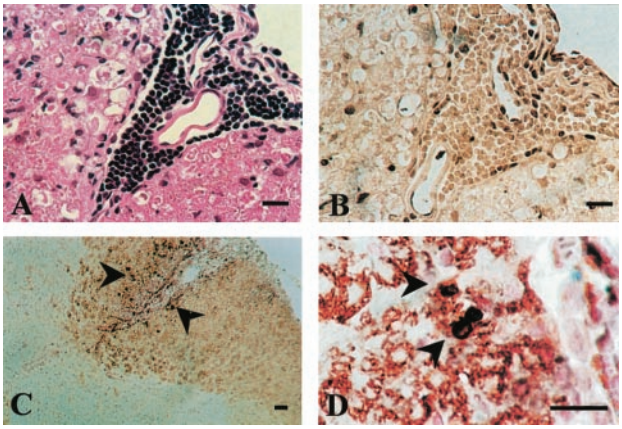

FIGURE 2. Apoptosis of OLG in acute EAE. *A*, Cervical spinal cord paraffin sections from C5-d mice with EAE show well-demarcated and confined lesions with inflammatory infiltrates and a narrow rim of demyelination (H&E). *B*, TUNEL$^+$ cells (dark-brown cells) are present within the inflammatory infiltrates and in deep white matter. *C* and *D*, Apoptotic OLG are identified on cryostat sections by double staining (arrowheads) using MAB 328 for OLG (red) and TUNEL assay for apoptosis detection (dark brown). Original magnifications: *A* and *B*, ×200; *C*, ×100; *D*, ×1000. Bar on each figure represents 10 μm.

and 3*A*). Cells with red cytoplasm (MAB 328) and a dark-brown nucleus (TUNEL) were identified as apoptotic OLG. Three consecutive sections of cervical spinal cords per animal were examined by double staining, and data were expressed as percentage of TUNEL$^+$ OLG in relation to total TUNEL$^+$ cells per section. Although apoptotic OLG were present in both strains of mice, they were significantly higher in C5-d compared with the C5-s mice both during the acute phase and recovery (Fig. 3*B*). These data suggested that OLG apoptosis was an integral part of the host inflammatory response in EAE, and this process appeared more prominent in C5-d mice.

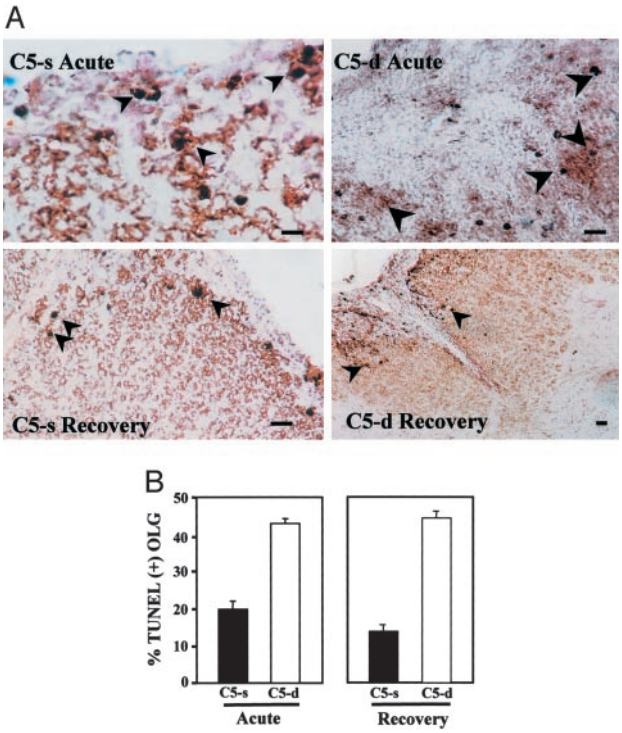

FIGURE 3. Apoptotic OLG during acute EAE and recovery phase. *A*, Cryostat sections were double stained for TUNEL (dark nuclei) and for OLG (red cytoplasm). Apoptotic OLG are seen predominantly along edges of the demyelinated area (arrowheads). Original magnifications: ×400, except for the *lower right panel* (×200). Bar on each figure represents 10 μm. *B*, Quantitative evaluation of apoptotic OLG was performed on C5-s and C5-d mice ($n = 4$, per each time point). Data are mean ± SEM, expressed as percentage of TUNEL$^+$ OLG relative to total TUNEL$^+$ cells counted. In C5-d mice, the number of apoptotic OLG was significantly higher than that of C5-d mice, both during acute EAE and recovery phases ($p < 0.04$).

Expression of FasL in C5-s mice with acute EAE

To gain insight into the possible role of C5 and/or MAC on apoptosis in EAE, we examined the expression of apoptosis-related genes in acute EAE using a gene array. Using the mRNA ratio to β-actin, only those expressing >3-fold higher in either strain of mice were considered significant. Of 96 genes, only 3 (*caspase 7*, *CD27*, and *FasL*) showed >3-fold higher expression in C5-s mice. Differential expression of FasL was confirmed by RT-PCR (Fig. 4*A*). By RT-PCR, FasL mRNA expression was significantly increased over the levels in control mice in both C5-s and C5-d (Fig. 4*A*). Increased expression of FasL in EAE was also confirmed by immunohistochemistry (Figs. 4*B* and 5, *A* and *B*). Therefore, a relatively small number of proapoptotic genes appeared to be differentially regulated in these strains of mice. The C5-dependent expression of FasL during EAE was a novel finding.

Expression of FasL and Fas proteins in EAE

Because Fas-mediated apoptosis has been reported to play an important role in remission and recovery of EAE (27) and FasL mRNA was differentially expressed in C5-s and C5-d, we examined FasL and Fas proteins in acute EAE and during recovery. More FasL$^+$ cells were seen in C5-s than in C5-d mice in acute EAE, whereas no significant difference was found in the recovery phase of EAE (Figs. 4*B* and 5, *A* and *B*). Whereas FasL was almost exclusively confined to inflammatory cells in all tissue sections studied (Fig. 5, *A* and *B*), cells expressing Fas in EAE were mostly noninflammatory cells located along the demyelinated borders (Fig. 6,

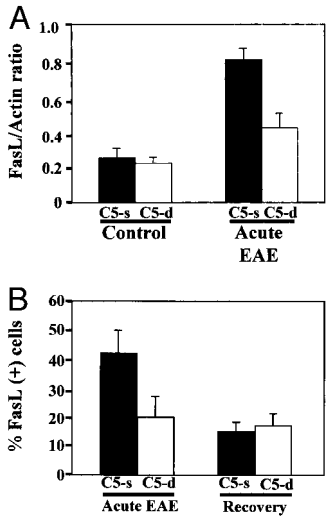

FIGURE 4. FasL expression in C5-d and C5-s mice with EAE. *A*, RT-PCR for FasL. The densitometry of radiographic bands obtained by RT-PCR is expressed as a ratio to β-actin and shown as a bar graph. FasL mRNA, constitutively expressed in control mice, is significantly increased in acute EAE. C5-s mice expressed significantly higher levels of FasL when compared with C5-d. *B*, Quantitation of FasL expression in C5-d and C5-s mice with EAE. Spinal cord cryostat sections from C5-d ($n = 3$) and C5-s ($n = 3$) mice during acute EAE and recovery were evaluated for FasL expression by immunostaining and expressed as mean ± SEM percentage of FasL$^+$ cells in relation to the total number of cells counted.

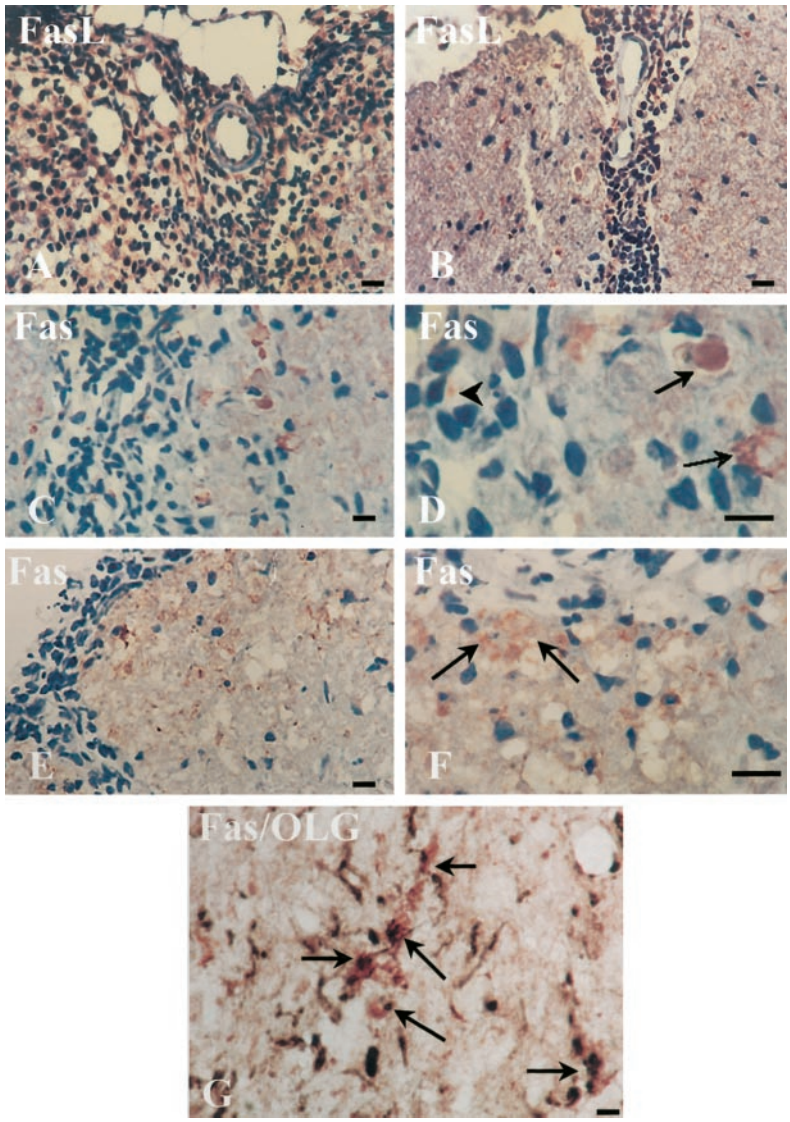

FIGURE 5. Tissue expression of FasL and Fas in EAE. *A–F*, Spinal cord cryosections were examined for FasL and Fas by indirect immunoperoxidase staining. FasL: In acute EAE, more cells in C5-s mice were FasL positive (*A*) when compared with C5-d mice (*B*). Fas: Immunostaining of Fas was mostly restricted to noninflammatory cells (arrow), and was rarely seen in inflammatory cells (arrowheads); some inflammatory cells are in close contact with Fas^+ cells (*C* and *D*). In C5-d mice during recovery, high-intensity staining of parenchymal cells for Fas is seen (*E*). Fas is expressed by many noninflammatory cells at the edge of demyelinated areas (*F*, arrows). *G*, Double staining in C5-s mice with acute EAE identified many of these Fas^+ parenchymal cells as OLG (arrows: dark-brown nucleus with red cytoplasm). *A* and *B*, ×200; *C*, *F*, and *G*, ×400; *D* and *E*, ×1000. Bar on each figure represents 10 μm.

E and *F*). Some of the Fas^+ cells were in close contact with inflammatory cells (Fig. 5, *C* and *D*). In acute EAE, a significantly higher number of Fas^+ cells was present in C5-s mice (57% of total number of cells counted) than in C5-d (39%) ($p < 0.003$) (Fig. 6). During recovery, Fas^+ cells significantly decreased in C5-s (7%), but much less decrease was found in C5-d mice (30%; Fig. 6) ($p < 0.001$). Because some of the Fas^+ cells showed morphologies resembling OLG, we examined tissue sections by double staining for Fas and OLG. As shown in Fig. 5, many Fas^+ OLG were found along the demyelinated border. These findings suggested that Fas expression on cell surfaces, including on OLG, might be regulated by inflammation associated with demyelination.

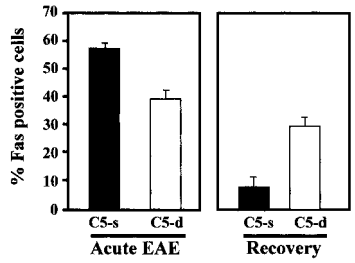

FIGURE 6. Quantitation of Fas expression in C5-d and C5-s mice with EAE. Spinal cords from C5-d ($n = 3$) and C5-s ($n = 3$) mice during acute EAE and recovery were evaluated for Fas expression by immunostaining and expressed as mean ± SEM percentage of Fas^+ cells in relation to the total number of cells counted.

Discussion

In this study, we investigated the role of C5 in apoptosis involving the CNS cells in EAE. Apoptosis involving both inflammatory cells and OLG was found in both acute EAE and remission, both in C5-s and C5-d mice. Total apoptotic cells were similarly increased in both strains of mice in acute EAE. However, apoptotic cells were significantly decreased in C5-s mice during recovery, a finding previously reported in SJL mice before remission of EAE (27). Apoptosis of inflammatory cells and their elimination are thought to be a critical determinant for the recovery of EAE (7, 28, 29). Therefore, the decrease in apoptotic inflammatory cells seen during recovery in C5-s, but not in C5-d-mice, may indicate a role for C5 in the clearance of inflammatory cells leading to a more efficient recovery from EAE. We examined the effect of C5 deficiency on apoptosis of OLG in these mice. In both acute EAE and recovery, the level of apoptotic OLG was significantly higher in C5-d mice than in C5-s mice. These results are consistent with our recent work showing near complete remyelination during the late

stage of EAE in C5-s but not in C5-d mice (24). Therefore, it is likely that C5 regulates the pathogenesis of EAE by contributing to the elimination of apoptotic inflammatory cells and promoting the recovery through OLG survival and remyelination. The role of C5 deficiency in neuronal cell death was examined in models of neurodegeneration using the same congenic pair of C5-s and C5-d mice (30). C5-d mice had more neuronal cell death, suggesting that C5 might be required to protect neurons from neurodegenerative stimuli. Our data are consistent with these findings and suggest that C5 is required also for protection of OLG from apoptosis in EAE.

As for the mechanisms by which C5 may decrease apoptotic inflammatory cells and OLG, the role of the Fas-FasL pathway was investigated. Increased expression of Fas on OLG was also seen in both acute and chronic EAE (29). Fas-mediated apoptosis represents an important mechanism in regulating the autoimmune response, and plays a critical role in remission following the acute inflammatory response in EAE (27, 31). Our data indicate that C5 may play a role in regulating Fas-FasL-mediated apoptosis during EAE. The expression of FasL and Fas were higher in acute EAE, followed by a decrease during remission in C5-s mice, whereas FasL and Fas^+ cells remained high in C5-d mice in remission. The up-regulation of FasL expression on inflammatory cells and Fas on OLG might help to explain the increased number of apoptotic OLG. The increase in apoptotic OLG in C5-d mice both in acute EAE and recovery also correlates with persistent increased Fas expression on these cells. Moreover, many Fas-positive OLG are localized in the area of active demyelination and in close contact with $FasL^+$ inflammatory cells (Fig. 5). These findings are significant and suggest that C5 deficiency may modify the OLG response to injury in part, through up-regulating Fas and promoting their apoptosis. Because C5a has no effect on acute EAE (25) or in enhancing OLG survival (20) in vitro and C5b participate in formation of MAC, the observed effect of C5 may also involve generation of MAC. Further studies using C6-deficient rats may clarify the role of MAC.

Despite the potential of MAC to cause OLG death by necrosis or apoptosis (1), OLG are relatively resistant to complement attack in vivo because of their ability to remove MAC from the cell surface (32) and, in some species, by their expression of inhibitory molecules like CD55 and CD59 (33). In addition, sublytic MAC may further enhance OLG survival from apoptosis induced by TNFα (20) or by a Fas-dependent process (5). However, apoptosis predominantly affecting OLG has not been a consistent finding in EAE and MS (5, 6, 11, 29, 34). Our finding that apoptosis of OLG is more prominent in C5-d mice is also consistent with those reports showing that apoptosis of OLG is a less prominent feature of EAE in animals with normal C5 (29).

We can therefore speculate that C5, and possibly the assembly of MAC, may regulate the inflammatory response of EAE and subsequent recovery. This may be achieved by protecting OLG from Fas-mediated apoptosis, thus promoting remyelination.

Acknowledgments

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