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TNF-α and αB-crystallin upregulation during antibody-mediated demyelination in vitro: a putative protective mechanism in oligodendrocytes.

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ABSTRACT

By using an in vitro model of antibody-mediated demyelination, we investigated the relationship between tumor necrosis factor-α (TNF-α) and heat shock protein (HSP) induction with respect to oligodendrocyte survival. Differentiated aggregate cultures of rat telencephalon were subjected to demyelination by exposure to antibodies against myelin oligodendrocyte glycoprotein (MOG) and complement. Cultures were analyzed 48 hr after exposure. Myelin basic protein (MBP) expression was greatly decreased, but no evidence was found for either necrosis or apoptosis. TNF-α was significantly up-regulated. It was localized predominantly in neurons and to a lesser extent in astrocytes and oligodendrocytes, and it was not detectable in microglial cells. Among the different HSPs examined, HSP32 and αB-crystallin were up-regulated; they may confer protection from oxidative stress and from apoptotic death, respectively. These results suggest that TNF-α, often regarded as a promoter of oligodendroglial death, could alternatively mediate a protective pathway through αB-crystallin up-regulation.

KEYWORDS

Multiple sclerosis; aggregating brain cell cultures; apoptosis; necrosis

INTRODUCTION

Multiple sclerosis (MS) and other demyelinating diseases of the central nervous system show different patterns of myelin destruction and oligodendrocyte survival, suggesting that several pathogenic mechanisms are involved in distinct subtypes of the disease (Lucchinetti et al., 1996). One potential demyelinating mechanism seems to be linked to the presence of autoantibodies. A pathogenic role for antibodies is suggested by observations showing that intact B-cell function is required for full expression of experimental allergic encephalomyelitis (EAE), an animal model for MS (Gausas et al., 1992), and that antibodies directed against specific CNS antigens, such as myelin oligodendrocyte glycoprotein (MOG), have demyelinating activity in vivo (Linington and Lassman, 1987) and in vitro (Honegger and Matthieu, 1990; Kerlero de Rosbo et al., 1990). Among the different myelin antigens, MOG is of particular interest, in that it induces both T-cell activation with a specific pattern of inflammation and antibody-induced demyelination (Adelmann et al., 1995). MOG is a minor myelin protein (less than 0.05% of total myelin proteins) with an immunoglobulin-like extracellular domain and is expressed on the outermost layer of the myelin sheath, accessible to antibody attack (Brunner et al., 1989; Amiguet et al., 1992). Autoantibodies against MOG have been identified within acute MS lesions, bound to disintegrating myelin membranes (Genain et al., 1999).

The fate of oligodendrocytes during inflammatory demyelinating processes, such as those occurring in MS, is still unsettled (Raine, 1997). In some MS lesions, precipitation of immunoglobulins and activated terminal complement components seem to be associated with oligodendrocyte survival and remyelination (Storch et al.,

1998). Several studies in vitro have shown that oligodendrocytes may undergo cell death by either necrosis or apoptosis (Selmaj et al., 1991). However, there is still controversy concerning oligodendrocyte apoptosis in MS: whereas in chronic progressive MS lesions no direct evidence of oligodendrocyte apoptosis was found (Bonetti and Raine, 1997), extensive apoptosis was observed in newly forming lesions of patients with relapsing and remitting MS (Barnett and Prineas, 2004). This suggests that different pathogenic mechanisms are involved in lesion formation (Kornek and Lassmann, 2003).

Tumor necrosis factor-α (TNF-α) has been shown to play a prominent role in CNS pathologies, including MS. In addition to its proapoptotic action mediated by the TNF receptor 1 (TNFR1), it is regarded as a multifunctional proinflammatory cytokine that induces a wide range of biological responses, including the activation of macrophages/microglia and the direct cytolysis of oligodendrocytes (for review see Selmaj and Raine, 1988; Merrill and Scolding, 1999). However, the exact pathogenic role of TNF-α in demyelinating processes remains controversial. Recently, EAE induced in TNF-α knockout mice has produced contradictory results indicating that TNF-α can exert both exacerbating and attenuating effects (Liu et al., 1998; Arnett et al., 2001; Matejuk et al., 2002). Moreover, clinical trials aimed at the neutralization of TNF-α in MS patients have shown little benefit or even aggravation of the clinical symptoms in some patients (van Oosten et al., 1996; The Lenercept Group, 1999). It therefore appears that, under certain circumstances, TNF-α can induce specific protective mechanisms in oligodendrocytes, analogous to those observed in neurons (Bruce et al., 1996). The heat shock response would be one example of these putatively protective reactions.

Heat shock proteins (HSPs) are produced in response to a wide variety of stressful insults and act as molecular chaperones that prevent inappropriate protein-protein interactions and aggregation of damaged or misfolded proteins (Hightower, 1991; Calabrese et al., 2002). Among HSPs, HSP70 and HSP90 are antiapoptotic by their direct inhibition of caspase activation (Beere et al., 2000; Pandey et al., 2000; Saleh et al., 2000). Interestingly, in astrocytes in vitro, TNF-α specifically regulates the expression of αB-crystallin, a member of the HSP family related to HSP27 (Bajramovic et al., 2000). αB-crystallin is constitutively expressed in many tissues, such as the lens, the heart, and the skeletal muscle (Clark and Muchowski, 2000). Its expression is strongly induced in different types of CNS injury, particularly in MS lesions (Bajramovic et al., 1997), where it is also recognized as a dominant T-cell antigen in myelin derived from MS patients (Van Noort et al., 1995). Because of its chaperone activity, αB-crystallin could protect oligodendrocytes during a demyelinating episode.

In the present study, the fate of oligodendrocytes was investigated in an established in vitro model of antibody-mediated demyelination, by using myelinated aggregating brain cell cultures and anti-MOG antibodies plus complement to induce demyelination (Honegger and Matthieu, 1990; Kerlero de Rosbo et al., 1990; Pouly et al., 2001; Besson Duvanel et al., 2001, 2003). By combining biochemical assays with immunocytochemistry and in situ hybridization, cultures were examined for cell death, changes in myelination, and expression and localization of TNF-α and HSPs, with special emphasis on HSP32 and αB-crystallin.

MATERIAL AND METHODS

Aggregating brain cell cultures

Fetal (16 days of gestation) rat telencephalons (OFA; Charles River Laboratories) were mechanically dissociated by using nylon sieves with 115- and 200-μm pores to prepare rotation-mediated aggregating brain cell cultures, as described previously (Honegger and Monnet-Tschudi, 2001). The aggregates were maintained in a serum-free medium (Honegger and Monnet-Tschudi, 2001) under constant rotation at 37°C and in an atmosphere of 10% CO_2 and 90% humidified air. Every 2 days, 5 ml of medium were exchanged per flask out of a total volume of 8 ml.

Antibody mediated demyelination

At culture day 27, the contents of each culture flask were split into four equal parts, and the final volume of each flask was made up to 8 ml by addition of fresh medium. Two sets of control cultures were established. The first set was kept untreated, the second challenged with complement alone. As a source of complement, we used guinea pig serum at a concentration of 25 μl/ml of culture medium. Demyelination was induced by adding to the cultures a mouse monoclonal antibody against rat MOG (62.5 μg/ml of culture medium) and complement for 48 hr. This antibody was derived from clone 8-18C5 (Linington et al., 1984); the immunoglobulin G (IgG) fraction was purified by affinity chromatography by using the Bio-Rad Econo-Pac protein A kit (Bio-Rad, Richmond, CA). For biochemical analyses, brain cell aggregates were washed twice with 5 ml ice-cold phosphate-buffered saline (PBS) and homogenized in 0.5 ml potassium phosphate buffer (2 mM, pH 6.8) containing 1 mM EDTA, with glass-glass homogenizers (Bellco, Vineland, NJ). The different homogenates were briefly sonicated and stored at -80°C as aliquots for the different assays. For

morphological analysis, aggregates were washed with PBS, embedded in tissue freezing medium (Jung, Nussloch, Germany), frozen in isopentane cooled with liquid nitrogen, and stored at -80°C, as described previously (Honegger and Monnet-Tschudi, 2001).

Biochemical assays

The protein concentration was measured by the Folin phenol method (Lowry et al., 1951) with bovine serum albumin as a standard. Radioimmunoassay was used to quantify the MBP content (Bürgisser, 1983). The 2′,3′-cyclic nucleotide 3′-phosphohydrolase (CNP) activity was measured by using a spectrophotometric technique (Kurihara and Tsukada, 1967). The activities of both choline acetyltransferase (ChAT), a marker for cholinergic neurons, and glutamic acid decarboxylase (GAD), a marker for γ-aminobutyric acid (GABA)-ergic neurons, were determined as described by Wilson et al. (1972). Glutamine synthetase (GS) activity, an astrocytic marker, was measured by a modified radioenzymatic method of Pishak and Phillips (Patel et al., 1982) with L-[1-^{14}C]glutamic acid as precursor and phosphoenolpyruvate/pyruvate kinase as the ATP-generating system.

Apoptosis/necrosis

The presence of necrotic cells was assessed by measuring the release of lactate dehydrogenase (LDH) into the culture medium by a conventional photometric technique (Pouly et al., 1997). Apoptosis was determined by two different techniques, namely, nucleosomic DNA laddering and terminal deoxynucleotidyl transferase tailing reaction (TUNEL). DNA fragmentation analysis was performed essentially as

described by Eizenberg et al. (1995). The aggregates were first washed twice in a solution of 140 mM NaCl and 20 mM EDTA, then incubated at 37°C in 400 µl lysis buffer (200 mM Tris-HCl, pH 8.3, 100 mM EDTA, 50 µg/ml proteinase K, and 1% sodium dodecyl sulfate). DNA was extracted twice with the conventional phenol/chloroform method. The aqueous phase containing the DNA was dialyzed overnight at 4°C against a solution of 10 mM Tris-HCl, pH 7.5, and 1 mM EDTA. This procedure was followed by a 3-hr incubation at 37°C with RNase A (50 µg/ml). Proteinase K (120 µg/ml) was added, and incubation was continued for another 3 hr at 37°C. DNA was again extracted twice with the conventional phenol/chloroform method and then precipitated in ethanol. Pellets were resuspended in 50 µl H_2O. DNA concentrations were measured by spectrophotometry at 260 nm. Five micrograms of each DNA sample were subjected to electrophoresis in a 1% agarose gel, and DNA fragments were visualized by staining with ethidium bromide.

TUNEL labeling was performed on cryostat sections fixed in a solution of PBS containing 4% paraformaldehyde, pH 7.4, for 20 min at room temperature. The sections were washed twice with PBS, incubated with a permeabilization solution (0.1% Triton X-100, 0.1% sodium citrate) for 2 min on ice, and rinsed twice in PBS. In accordance with the manufacturer's instructions (Roche Diagnostics, Mannheim, Germany), 50 µl TUNEL reaction mixture containing fluorescein-dUTP was added to the samples and incubated for 1 hr at 37°C in the dark. For negative controls, the terminal transferase step was omitted. Sections were then washed twice with PBS, mounted, and analyzed with a fluorescent microscope (Leitz).

In situ hybridization

Digoxigenin-labeled MBP riboprobes were transcribed in vitro from linearized, gel-purified plasmids by using T7 RNA polymerase as described previously (Tosic et al., 2002). Cryostat sections were hybridized for 40 hr at 58°C with digoxigenin-labeled probes (400 ng/ml) in 5× SSC (NaCl 0.75 M, Na-citrate 0.075 M) containing 50% formamide and 40 μg/ml salmon sperm DNA (Braissant, 2004). The sections were washed three times in decreasing concentrations of SSC and incubated with alkaline phosphatase-coupled antidigoxigenin antibody (5,000-fold dilution). The mRNAs were visualized by alkaline phosphatase staining. Sections were then dehydrated, mounted, and photographed with a light microscope (Leitz).

Gel electrophoresis and immunoblotting

A solution of antiprotease (Complete Mini; Boehringer/Roche, Indianapolis, IN) was added to the homogenized brain cell aggregates. Samples (80 μg) were precipitated in a fivefold volume of ice-cold acetone at -80°C during 2 hr. After a 30-min centrifugation (12,000 rpm at 4°C), the homogenates were solubilized in Laemmli buffer (1:1) and loaded on standard (12% w/v acrylamide/0.2% w/v bisacrylamide) polyacrylamide gels. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was then carried out. After separation, gels were blotted onto PVDF membranes and blocked for 2 hr at 37°C with 5% nonfat milk in Tris-buffered saline (TBS) containing 0.05% (v/v) Tween-20. The immobilized proteins were detected after an overnight incubation at 4°C with appropriate antibodies: anti-MOG (1,000-fold dilution), anti-TNF-α (500-fold dilution), anti-αB crystallin (1,000-fold dilution), antiheme oxygenase 1 (500-fold dilution; Sigma, St. Louis, MO), anti-HSP27 (500-fold dilution), anti-HSP70 (500-fold dilution), anti-HSP90 (1,000-fold dilution), and

antiactin (500-fold dilution; Sigma) as an internal control. Antibodies against αB-crystallin, TNF-α, HSP27, HSP70, and HSP90 were purchased from Calbiochem (Darmstadt, Germany). The blots were then washed three times for 5 min each in TBS-Tween. The appropriate species-specific horseradish peroxidase-conjugated secondary antibody (10,000-fold dilution) was applied for 1 hr at room temperature. After three 5-min washes in TBS-Tween, the signal was detected by using an enhanced chemiluminescence system (Amersham Pharmacia Biotech, Piscataway, NJ). The blots were exposed on BioMax MR-1 films (Kodak, Rochester, NY); then, the films were scanned on ImageScanner (Amersham Pharmacia Biotech), and semiquantitation was performed with the software analysis system TotalLab (Amersham Pharmacia Biotech).

Immunohistochemistry

Double labeling of cryostat sections was performed to identify the cell type(s) expressing TNF-α and αB-crystallin.

Double colorimetric immunohistochemistry

Cryostat sections (8 μm) were fixed 1 hr with 4% paraformaldehyde (PFA) in PBS. Endogenous peroxidase activity was bleached with 1.5% H_2O_2 for 15 min. After blockade of nonspecific binding with 1% bovine serum albumin (BSA) in PBS, sections were exposed 1 hr to rabbit polyclonal antibodies against TNF-α (1:30; Biosource International) or αB-crystallin (1:400; Calbiochem) diluted in PBS containing 1% BSA. After being washed, sections were processed using the Histostain-Plus kit according to the manufacturer's protocol (Zymed Laboratories, South San Francisco, CA): Sections were incubated with a biotinylated anti-rabbit

IgG secondary antibody, followed by a streptavidin-peroxidase conjugate. Peroxidase staining was performed for 30 min with aminoethyl carbazole and H_2O_2 (red signal) and was stopped in distilled water. To identify oligodendrocytes expressing TNF-α or αB-crystallin, some of the cryosections were subjected to a second immunohistochemical procedure for the labeling of the oligodendrocytic marker galactocerebroside (Gal-C). After blockade of nonspecific binding with 1% BSA in PBS, sections were exposed 1 hr to a mouse monoclonal antibody against Gal-C (1:20; Chemicon, Temecula, CA) diluted in 1% BSA in PBS. After being washed, sections were incubated for 1 hr with a goat anti-mouse IgG antibody coupled to alkaline phosphatase (1:500; Bio-Rad). Sections were finally stained (15 min) with nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl-phosphate (BCIP; blue signal). Levamisole (1%) was used in the staining buffer to block endogenous alkaline phosphatase activity. The labeling was stopped in 10 mM Tris containing 1 mM EDTA. Sections were mounted under glycerol and observed and photographed on an Olympus BX50 microscope equipped with a DP-10 digital camera (Olympus Opticals).

Double confocal immunofluorescence

Cryostat sections (10 µm) were fixed for 10 min in acetone at room temperature and dried overnight at 4°C (Honegger and Monnet-Tschudi, 2001). For blockade of nonspecific binding, sections were first incubated with a mixture of goat serum and donkey serum (1:20 in PBS with 0.1% Triton X-100; Jackson Immunoresearch, West Grove, PA) and exposed overnight at 4°C with polyclonal antibodies against either αB-crystallin (1:200; Calbiochem) or TNF-α (1:15; Biosource International) and with cell specific antibodies: Monoclonal antibodies against microtubule-associated protein

2 (MAP-2; 1:125; Biomarker) or synaptophysin (1:25; Chemicon), two markers for neurons; glial fibrillary acidic protein (GFAP; 1:200; Sigma), a marker for astrocytes; or MBP (1:200; Chemicon), a marker for oligodendrocytes. Isolectin B4 of *Griffonia simplicifolia* bound to fluorescein isothiocyanate (IB4 bound to FITC; 1:20; Sigma) was used as a marker for microglia. With polyclonal antibodies, the staining was developed with the avidin-biotin system [goat anti-rabbit biotinylated IgG; 1:100; FITC-labeled avidin (DCS); 1:50, Vector, Burlingame, CA]. With monoclonal antibodies, staining was obtained with donkey anti-mouse IgG bound to Cy3 (1:250; Jackson Immunoresearch). Monoclonal antibodies applied together with IB4 bound to FITC were stained with donkey anti-mouse IgG bound to Cy3. Sections were mounted in Fluosave Reagent (Calbiochem) and analyzed with a Leica confocal microscope. For one final image, 10 confocal images taken at steps of 1 μm were superposed. To verify a nuclear localization of αB-crystallin, double-labeled sections were incubated for 30 min at 37°C in a solution of 4′,6-diamidino-2-phenylindole (DAPI; 1 mg/ml; Sigma). Sections were observed with a Leica fluorescence microscope with appropriate filters.

RESULTS

Anti-MOG antibodies and complement caused demyelination without a significant increase of cell death

In myelinated aggregating rat brain cell cultures, the addition of anti-MOG antibodies and complement significantly decreased the levels of MOG within 48 hr (Fig. 1, lane 3). Similarly, CNP activity taken as a marker of the uncompacted oligodendroglial membranes and MBP content as a marker of mature myelin were significantly decreased (Table I). MBP was also significantly decreased at the transcriptional level as shown by in situ hybridization (Fig. 2). Throughout, comparisons were made with untreated control cultures as well as with cultures treated with complement alone, the latter for the evaluation of possible toxic effects of complement on oligodendrocytes (Scolding et al., 1990). As shown in Table I, complement alone decreased both MBP content and CNP activity, although not to a statistically significant extent. The reason for these effects remain obscure, whether they are due to the inherent toxicity of complement or of other components in the guinea pig serum, which served as a source of complement. In contrast to the oligodendroglial markers, the presence of complement alone increased the levels of ChAT, GAD, and GS specific activities (Table I), as was found previously (Kerlero de Rosbo et al., 1990; Besson Duvanel et al., 2001). These stimulatory effects may be attributed to the presence of trophic factors in the guinea pig serum, as discussed previously (Kerlero de Rosbo et al., 1990).

Measurements of the LDH activity in treated cultures and in untreated controls showed no significant variation (Table I), indicating that neither the addition of complement alone nor the combination of complement and anti-MOG antibodies

caused necrotic cell death. Furthermore, the absence of nucleosomic DNA laddering (Fig. 3) indicated that the apoptotic cell death was not increased either. In accordance with these findings, no evidence for apoptotic death was found in the TUNEL labeling assay or in the Western blot analysis of p53 (results not shown).

Upregulation of TNF-α during demyelination and cellular localization of TNF–α

Aggregate cultures subjected to demyelination were examined for TNF-α expression by Western blot analysis (Fig. 4) and immunohistochemistry (Fig. 5). The results show that, under demyelinating conditions, TNF-α was up-regulated (Fig. 4, lane 3; Fig. 5c) compared with the basal levels found in untreated controls (Fig. 4, lane 1; Fig. 5a) and those found in cultures treated with complement alone (Fig. 4, lane 2; Fig. 5b). The latter indicated that complement alone did not affect the levels of TNF-α. The cellular expression of TNF-α was further examined by double immunostaining (Fig. 5d-l). TNF-α was always located in oligodendrocytes (Fig. 5d-f, colabeled for Gal-C): in untreated controls (Fig. 5d), after treatment with complement alone (Fig. 5e), and after treatment with complement and anti-MOG antibodies (Fig. 5f). The presence of TNF-α in oligodendrocytes was also demonstrated by colabeling for MBP (not shown). However, TNF-α was expressed mainly in astrocytes (Fig. 5g-i, colabeled for GFAP) and in neurons (Fig. 5j-l, colabeled for synaptophysin). TNF-α was not detectable in microglial cells (not shown).

Upregulation of αB-crystallin and HSP32 during demyelination and cellular localization od αB-crystallin

Analysis by Western blotting of the expression of HSPs showed that, of the five HSPs tested (i.e., HSP32, HSP27, HSP70, HSP90, and αB-crystallin), only two (HSP32 and αB-crystallin) were specifically up-regulated by the anti-MOG-induced

demyelination (Fig. 6). HSP32 was found at low levels in untreated cultures (Fig. 6, lane 1). Its expression was increased after the addition of complement (Fig. 6, lane 2) and further increased in demyelinating cultures (Fig. 6, lane 3). αB-crystallin was weakly expressed in untreated controls (Fig. 6, lane 1) and in complement-treated cultures (Fig. 6, lane 2), whereas it was strongly up-regulated under demyelinating conditions (Fig. 6, lane 3). In contrast, no change was found for HSP 90 (Fig. 6) and HSP 70 (not shown), but HSP27 was slightly, but not significantly, decreased in the treated cultures (Fig. 6).

At the histological level, αB-crystallin was also found to be up-regulated in demyelinating cultures (Fig. 7c) compared with untreated controls (Fig. 7a) and cultures treated with complement alone (Fig. 7b). As for TNF-α (Fig. 4, 5) and as observed by Western blotting (Fig. 6), no effect of complement alone could be observed for αB-crystallin at the histological level. αB-crystallin was further analyzed at the cellular level by double immunostaining (Fig. 7d-l). As for TNF-α, αB-crystallin was demonstrated in oligodendrocytes by colabeling for Gal-C (Fig. 7d-f) and MBP (not shown) in untreated controls (Fig. 7d), after treatment with complement alone (Fig. 7e), and after treatment with complement and anti-MOG (Fig. 7f). However, most of the αB-crystallin expression was localized in astrocytes (Fig. 7g-l, colabeled for GFAP), where a punctated staining was observed in the nuclei both in untreated controls (Fig. 7g-i) and in demyelinating cultures (Fig. 7j-l, observed after lowering of the FITC signal threshold). The nuclear localization of αB-crystallin was further confirmed with DAPI as nuclear dye (not shown). The increased staining of GFAP observed in demyelinating cultures (Fig. 7k compared with h) suggests that, under these conditions, the astrocytes were in a reactive state.

DISCUSSION

For MS and EAE, several demyelinating mechanisms have been proposed, including the direct immune attack against specific myelin components (Brosnan and Raine, 1996), activation of cytokine receptors of oligodendrocytes (Dowling et al., 1996), and generation of reactive oxygen and nitrogen intermediates (Merrill and Benveniste, 1996). Strong evidence for autoimmune antibody-mediated demyelination stems from the detection of anti-MOG immunoglobulins associated with myelin damage in MS (Genain et al., 1999). MOG is of particular interest, in that it is so far the only myelin antigen known that elicits both a T-cell-mediated inflammatory immune response and antibody-mediated demyelination (Adelmann et al., 1995). With respect to the MS lesions showing oligodendrocyte survival and remyelination, it is noteworthy that they are associated with local precipitation of immunoglobulins and activated terminal complement components (Storch et al., 1998). These observations suggest that antibody-mediated demyelination induces secondary reactions that may attenuate further immunological attacks.

The fate of oligodendrocytes during demyelination is still a matter of debate (Raine, 1997). Although the apoptotic death of oligodendrocytes has been observed under various circumstances (Selmaj et al., 1991; Casaccia-Bonnefil, 2000), its occurrence in MS lesions has been questioned (Bonetti et al., 1997; Barnett and Prineas, 2004). In the present study, there was no sign of apoptosis after antibody-mediated demyelination in three-dimensional brain cell cultures, despite the drastic decrease of myelin-specific markers. The concomitant reduction in MBP mRNA levels may

indicate dedifferentiation of oligodendrocytes, in accordance with previous observations after phorbolester-induced demyelination (Pouly et al., 1997).

Among the potential mediators of MS pathogenesis, the proinflammatory cytokine TNF-α has often been associated with oligodendrocyte injury and cell death (D'Souza et al., 1995). However, its exact role in oligodendrocyte injury and demyelination remains unclear. One of the reasons may be the existence of two different TNF-α receptors, TNFR1 (p55) and TNFR2 (p75; Brockhaus et al., 1990). Both receptors have been implicated in apoptotic mechanisms, although TNFR1 appeared as the principal mediator of cell death, whereas TNFR2 also promoted cell activation, growth, and proliferation (Nagata, 1997). There are diverging reports concerning the expression of TNFR1 and/or TNFR2 by oligodendrocytes in vivo and in vitro (for review see Hisahara et al., 2003). In any case, evidence for TNFR1-mediated apoptosis in vivo is lacking (Bonetti and Raine, 1997). In the present in vitro model of MOG antibody-mediated demyelination, TNF-α expression was up-regulated. Whether it was also released by the cells could not be determined; the inmmunohistochemical approach did not allow to differentiate between intracellular and extracellularly bound TNF-α. At any rate, despite the up-regulation and possible release of TNF-α, there was no apparent change in the viability of the oligodendrocyte population. In EAE models, a prolonged increase of TNF-α production has been described for neurons (Villarroya et al., 1997; Shin et al., 1999), suggesting a protective role in neurons. TNF-α may protect neurons from axonal degeneration by the modulation of synaptic activity (Elenkov et al., 1992), adrenocorticotropic hormone (ACTH) secretion (Sharp et al., 1989), and/or nerve growth factor synthesis (Gadient et al., 1990).

Could antiapoptotic pathways be specifically triggered in oligodendrocytes during demyelination? Recent studies have demonstrated that HSP70 and HSP90 impede caspase activation (Beere et al., 2000; Pandey et al., 2000; Saleh et al., 2000). However, these two HSPs remained unchanged during the anti-MOG-induced demyelination, whereas HSP32 and αB-crystallin were up-regulated. HSP32, also known as *heme oxygenase 1*, belongs to a family of proteins that catalyze the oxidative degradation of heme to biliverdin. Heme increases the formation of reactive oxygen intermediates and, hence, exacerbates intracellular oxidative stress (Schipper, 2000). HSP32 is up-regulated in oligodendrocytes after an oxidative stress (Goldbaum and Richter-Landsberg, 2001), but it remains controversial whether an increased HSP32 level confers protection or rather aggravates cell damage (Schipper, 2000). At any rate, the present finding that HSP32 was strongly up-regulated during antibody-mediated demyelination indicates the involvement of oxidative stress in the pathogenesis of demyelination. αB-crystallin is related to HSP27, a member of the family of small HSPs. Small HSP members contain a highly conserved domain known as the α-*crystallin core*, located in the C-terminal part of the protein (Clark and Muchowski, 2000). They form oligomeric complexes with partners of the same family that function as molecular chaperones to facilitate protein folding and to prevent aggregation of denatured or misfolded proteins. Recently, αB-crystallin was reported to inhibit a downstream effector of the death receptor pathway and to regulate apoptosis in the cell negatively (Kamradt et al., 2001). Thus, αB-crystallin could be able to prevent TNF-α-induced apoptosis. Binding of TNF-α to its receptor recruits and activates procaspase-8 via a series of protein-protein interactions (Garrido et al., 2001). Active caspase-8 cleaves procaspase-3 to generate a p24 intermediate. α

B-crystallin inhibits the autoproteolytic formation of p24 necessary for the generation of active caspase-3, the final trigger of apoptosis in the cell. During MOG antibody-mediated demyelination in the aggregates, αB-crystallin was strongly up-regulated. Although the levels of HSP27, its oligomeric partner for molecular chaperone activity, decreased slightly during demyelination, sufficient protein might have been available to contribute to an antiapoptotic effect.

The up-regulation of αB-crystallin during demyelination may be directly linked to the up-regulation of TNF-α, in that the latter is the only cytokine reported to up-regulate specifically the expression of αB-crystallin in cultured astrocytes (Bajramovic et al., 2000). In aggregate cultures, αB-crystallin is up-regulated during demyelination in astrocytes and oligodendrocytes. Furthermore, a nuclear location was found for αB-crystallin in astrocytes, similar to that reported by Bajramovic et al. (2000). Taken together, the present results suggest that, during antibody-mediated demyelination, TNF-α triggered a protective pathway for oligodendrocytes via up-regulation of αB-crystallin.

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Table I. Effects of Anti-MOG Antibodies and Complement on Myelin, Oligodendrocytes, Cholinergic and GABAergic Neurons, Astrocytes, and Extracellular LDH Release†

	Control cultures	Complement (25 µl/ml)	Anti-MOG (62.5 µg/ml) and complement
Total protein (mg/flask)	2.6 ± 0.2	2.95 ± 0.2	2.95 ± 0.2
MBP (µg/mg total protein)	0.64 ± 0.05	0.52 ± 0.04	0.18 ± 0.02*
CNP (µmol of 2′-NADP · min^{-1}/mg total protein)	3.52 ± 0.4	2.39 ± 0.3	1.83 ± 0.23*
ChAT (pmol · min^{-1}/mg total protein)	355.2 ± 49.2	529.3 ± 56.0	410.8 ± 58.9
GAD (nmol · min^{-1}/mg total protein)	1,103.6 ± 111.8	1,565.3 ± 77.1	1,499.8 ± 95.2
GS (µmol · min^{-1}/mg total protein)	1.99 ± 3.1	2.45 ± 1.8	2.35 ± 2.5
LDH (µmol · min^{-1}/flask)	0.26 ± 0.01	0.29 ± 0.01	0.27 ± 0.01

† Data are mean ± SEM from five independent experiments.

* $P < 0.01$ (ANOVA and Dunnet's posttest).

LEGENDS

Fig. 1 Demyelination-induced down-regulation of MOG in aggregating rat brain cell cultures. Western blot analysis of MOG and actin in untreated controls (lane 1), in cultures treated with complement alone (lane 2), and in anti-MOG- and complement-treated aggregates (lane 3). Densitometric quantitation of MOG of blotted homogenates. Actin was used as an internal standard to correct for the difference in protein amounts. Data are mean ± SEM (n = 5). *$P < 0.05$.

Fig. 2 Demyelination-induced decrease in the expression of MBP mRNA. The transcriptional activity of the MBP gene in aggregates was detected by in situ hybridization. No signal was observed with the sense probes that were used as an internal control to ensure specific hybridization of the MBP mRNA (not shown). Untreated control culture (**a,d**), complement (**b,e**), and anti-MOG and complement (**c,f**); d-f are higher magnifications of a-c, respectively.

Fig. 3 Absence of apoptosis in aggregating brain cell cultures after antibody-mediated demyelination. Detection of apoptotic cells was done by DNA laddering. DNA was extracted from untreated control cultures (lane 1), complement-treated cultures (lane 2), anti-MOG- and complement-treated aggregates (lane 3), and a negative control of nonapoptotic primary cultures of oligodendrocytes (lane 4). A DNA molecular weight marker was also loaded. The bands were visualized by using ethidium bromide under UV exposure.

Fig. 4 Demyelination-induced up-regulation of TNF-α in aggregating rat brain cell cultures. Western blot analysis of TNF-α in untreated controls (lane 1), in cultures

treated with complement alone (lane 2), and in anti-MOG- and complement-treated aggregates (lane 3). A densitometric quantitation of TNF-α of blotted homogenates was made. Actin was used as an internal standard to correct for the difference in protein amounts. Data are mean ± SEM (n = 5). *$P < 0.05$.

Fig. 5 Immunohistochemistry showing the demyelination-induced up-regulation of TNF-α and the cellular expression of TNF-α in demyelinating brain cell aggregate cultures. TNF-α labeling with peroxidase (a-c), control conditions (a), complement alone (b), anti-MOG antibody and complement (c). Cellular localization of TNF-α by double colorimetric immunohistochemistry (d-f) and double immunofluorescence and confocal microscopy (g-l). Control conditions (d,g-l), complement alone (e), anti-MOG antibody and complement (f). Localization of TNF-α (red signal) in oligodendrocytes colabeled for Gal-C (blue signal; d-f). Localization of TNF-α (FITC; g) in astrocytes colabeled for GFAP (Cy3; h), and merged (i). Localization of TNF-α (FITC; j) in neurons colabeled for synaptophysin (Cy3; k) and merged (l). Scale bar in c = 100 μm for a-c; bar in f = 25 μm for d-f; bar in l = 10 μm for g-l.

Fig. 6 Western blot analyses of different HSPs and semiquantitation of αB-crystallin, HSP32, and actin. αB-crystallin and HSP32 were up-regulated after antibody-mediated demyelination. **A:** Western blot analysis of different HSPs using antibodies to HSP90, HSP32, actin, HSP27, αB-crystallin in untreated controls (lane 1), in cultures treated with complement alone (lane 2), and in anti-MOG- and complement-treated aggregates (lane 3). **B:** Densitometric quantitation of αB-crystallin (open bars) and HSP32 (solid bars) of blotted homogenates (A). Actin was used as an internal

standard to correct for the difference in protein amounts. Data are mean ± SEM (n = 3). *$P < 0.05$.

Fig. 7 Immunohistochemistry showing the demyelination-induced up-regulation of αB-crystallin and the cellular expression of αB-crystallin in demyelinating brain cell aggregate cultures. αB-crystallin labeling with peroxidase (a-c), control conditions (a), complement alone (b), anti-MOG antibody and complement (c). Cellular localization of αB-crystallin by double colorimetric immunohistochemistry (d-f) and double immunofluorescence and confocal microscopy (g-l). Control conditions (d,g-i), complement alone (e), anti-MOG antibody and complement (f,j-l). Localization of αB-crystallin (red signal) in oligodendrocytes colabeled for Gal-C (blue signal; d-f). Localization of αB-crystallin (FITC; g,j) in astrocytes colabeled for GFAP (Cy3; h,k) and merged (i,l). Scale bar in c = 100 μm for a-c; bar in f = 25 μm for d-f; bar in l = 10 μm for g-l.

Figure 1:

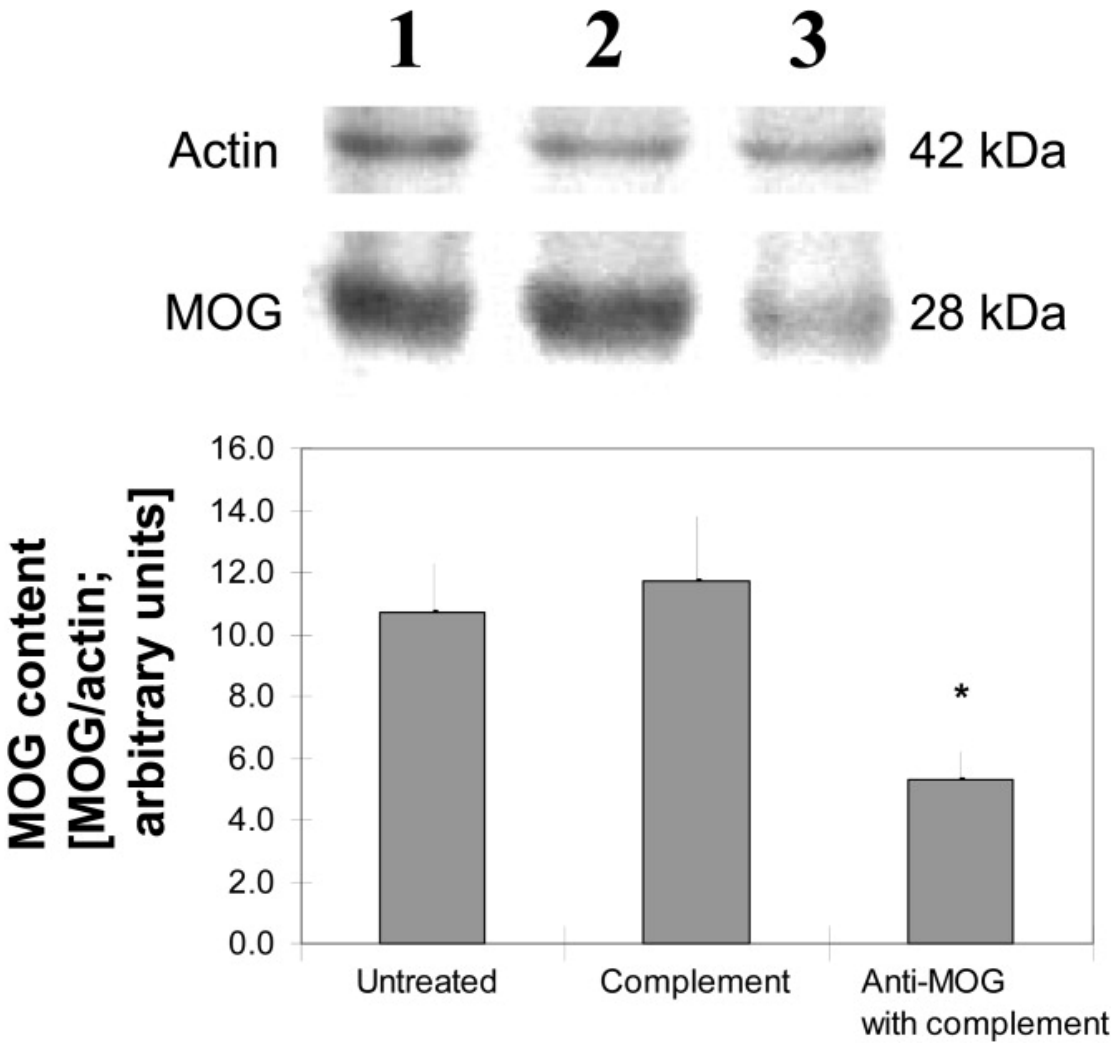
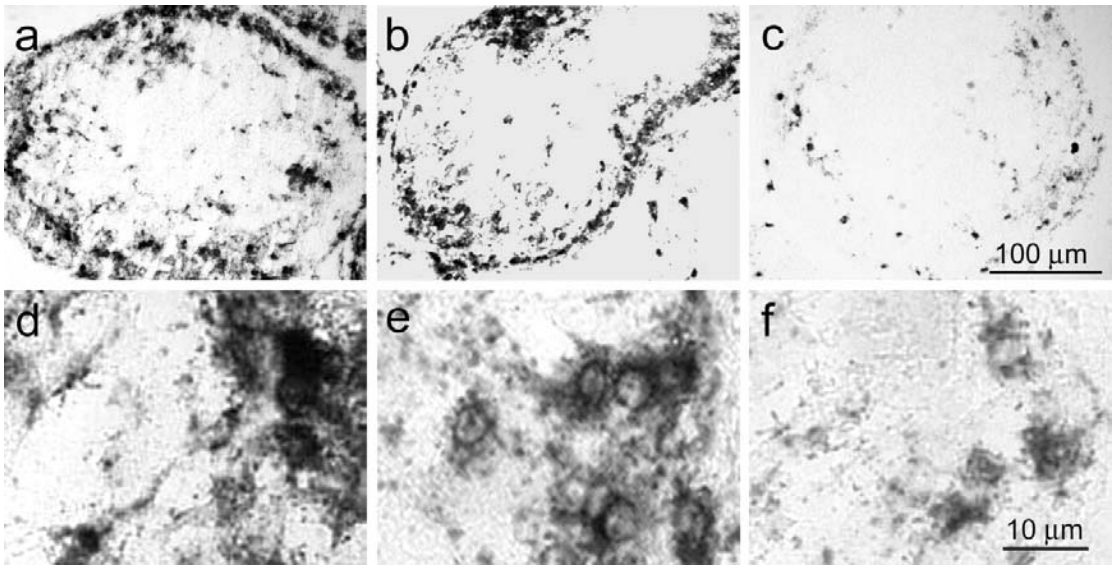


Figure 2:



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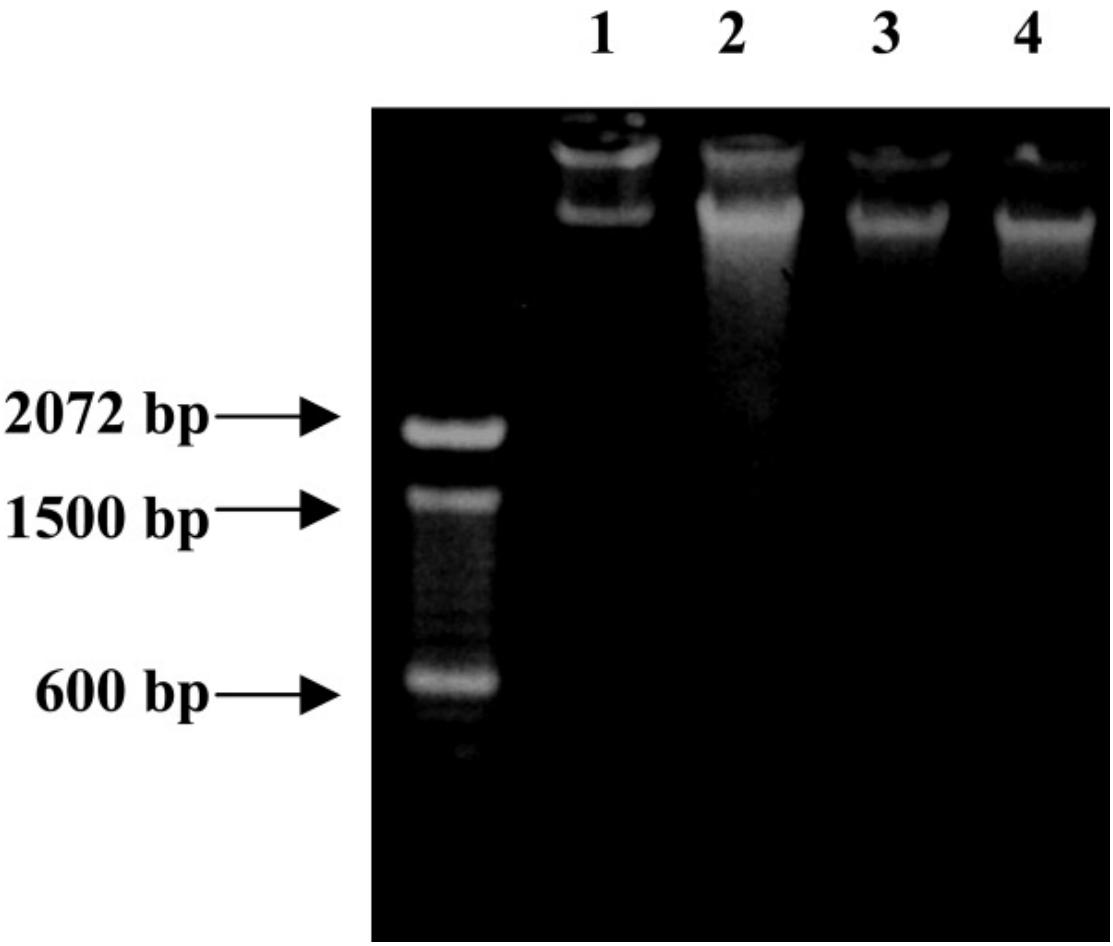

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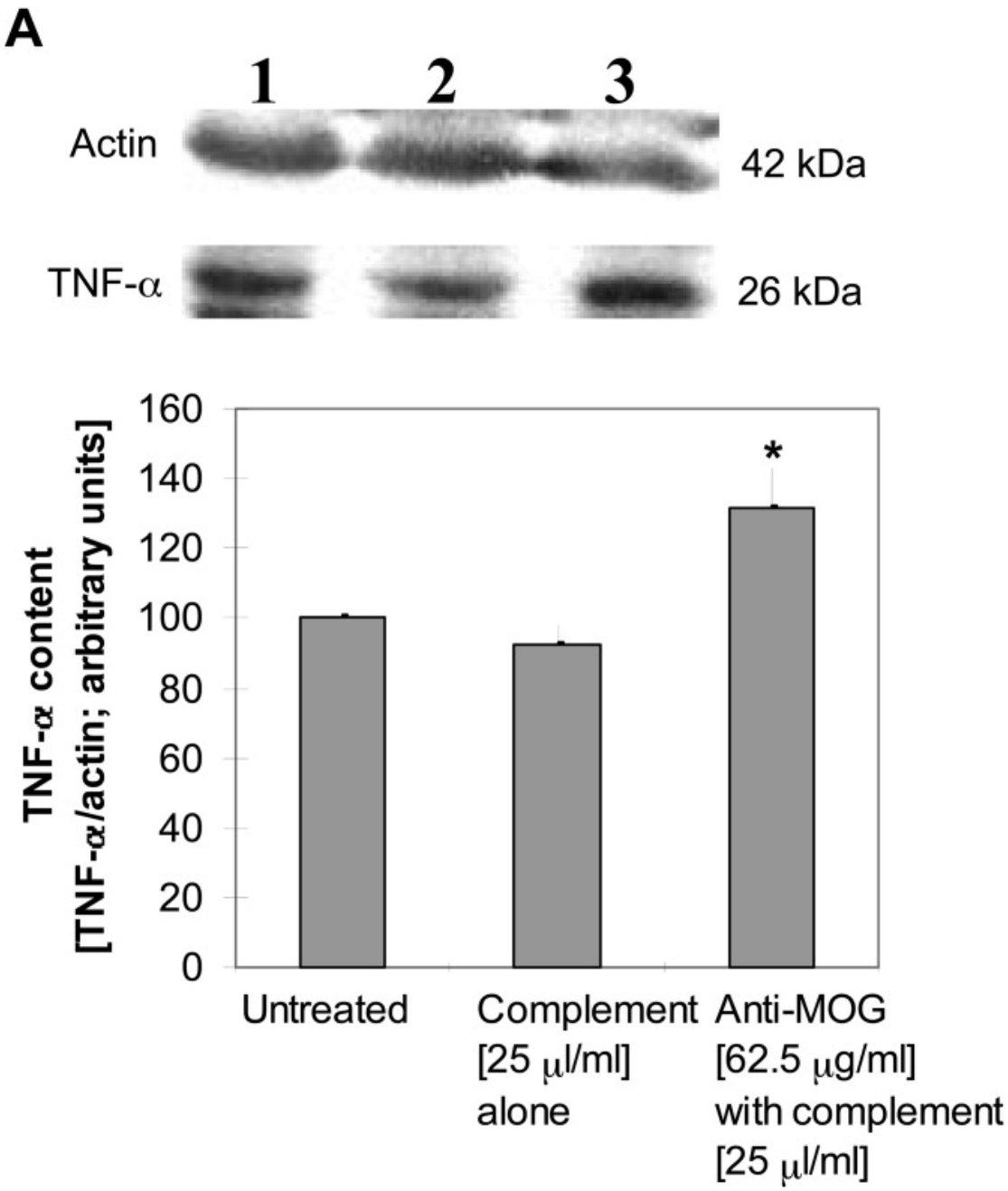

A
1
2
3
Actin
42 kDa
TNF-α
26 kDa
TNF-α content
[TNF-α/actin; arbitrary units]
160
140
120
100
80
60
40
20
0
*
Untreated
Complement
[25 μl/ml]
alone
Anti-MOG
[62.5 μg/ml]
with complement
[25 μl/ml]

Figure 5:

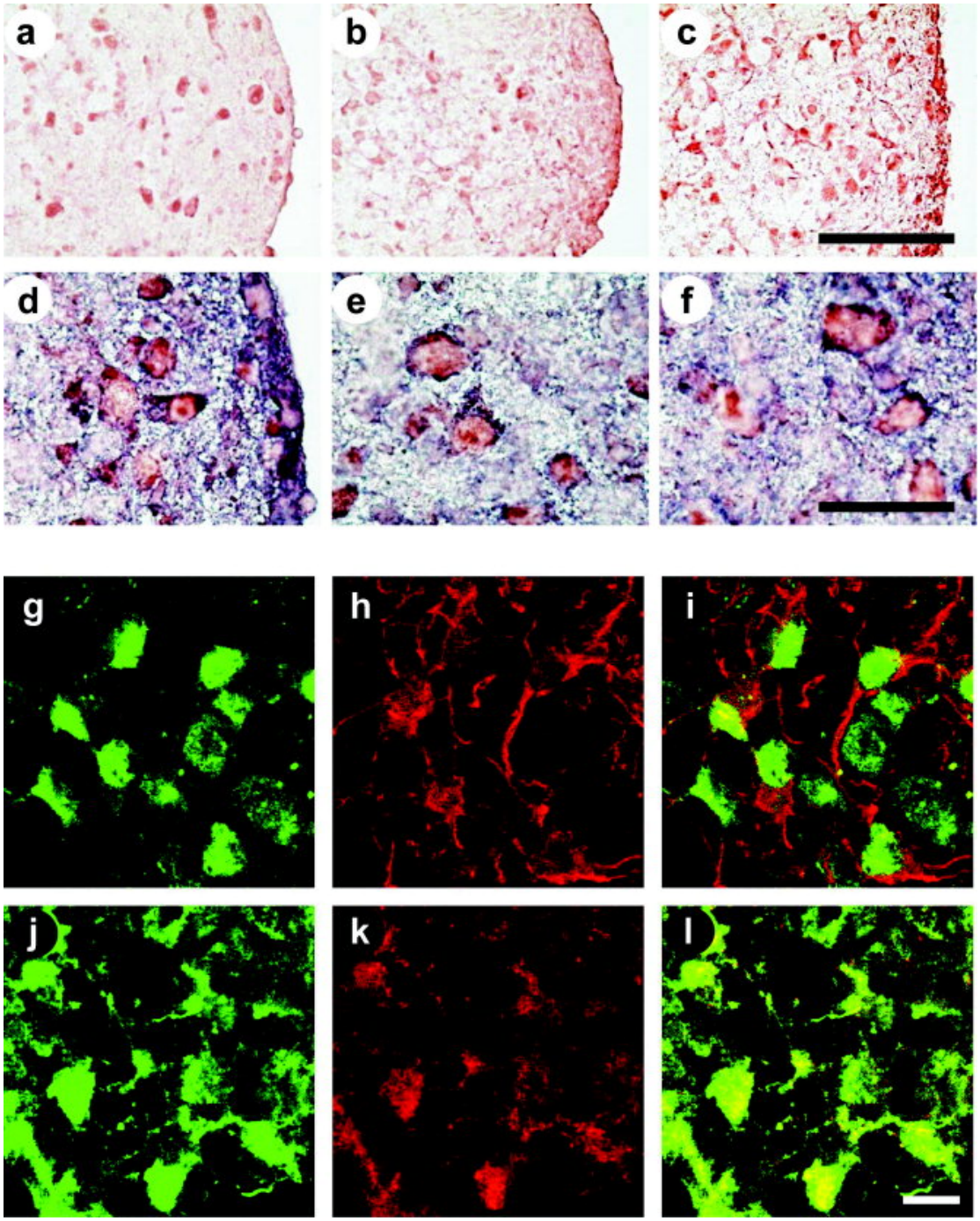
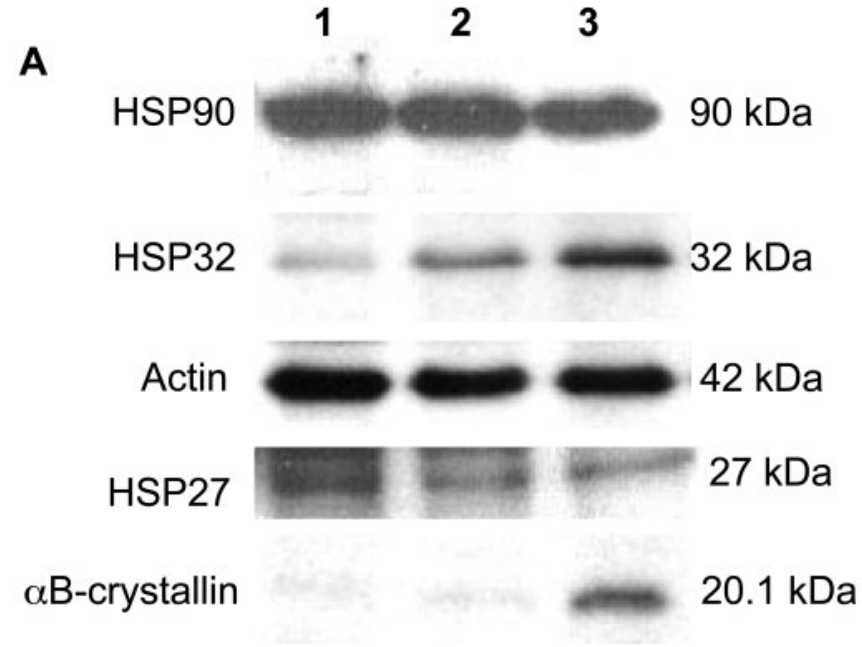


Figure 6:


A
1
2
3
HSP90
90 kDa
HSP32
32 kDa
Actin
42 kDa
HSP27
27 kDa
αB-crystallin
20.1 kDa

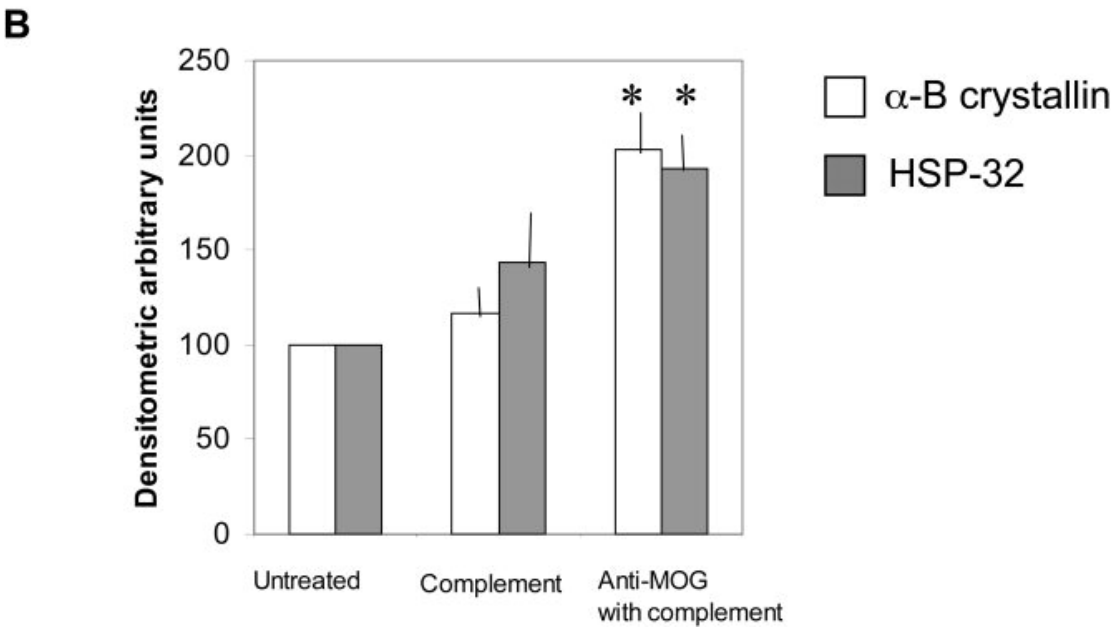

B
Densitometric arbitrary units
250
200
150
100
50
0
* *
α-B crystallin
HSP-32
Untreated
Complement
Anti-MOG
with complement

Figure 7: