Simultaneous neuroprotection and blockade of inflammation reverses autoimmune encephalomyelitis

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Summary
In multiple sclerosis, the immune system attacks the white matter of the brain and spinal cord, leading to disability and/or paralysis. Myelin, oligodendrocytes and neurons are lost due to the release by immune cells of cytotoxic cytokines, autoantibodies and toxic amounts of the excitatory neurotransmitter glutamate. Experimental autoimmune encephalomyelitis (EAE) is an animal model that exhibits the clinical and pathological features of multiple sclerosis. Current therapies that suppress either the inflammation or glutamate excitotoxicity are partially effective when administered at an early stage of EAE, but cannot block advanced disease. In a multi-faceted approach to combat EAE, we blocked inflammation with an anti-MAdCAM-1 (muco-sal addressin cell adhesion molecule-1) monoclonal antibody and simultaneously protected oligodendrocytes and neurons against glutamate-mediated damage with the N-terminal tripeptide of insulin-like growth factor). Remarkably, administration at an advanced stage of unremitting EAE of either a combination of NBQX and GPE, or preferably all three latter reagents, resulted in amelioration of disease and repair of the CNS, as assessed by increased oligodendrocyte survival and remyelination, and corresponding decreased paralysis, inflammation, CNS apoptosis and axonal damage. Each treatment reduced the expression of nitric oxide and a large panel of proinflammatory and immunoregulatory cytokines, in particular IL-6 which plays a critical role in mediating EAE. Mice displayed discernible improvements in all physical features examined. Disease was suppressed for 5 weeks, but relapsed when treatment was suspended, suggesting treatment must be maintained to be effective. The above approaches, which allow CNS repair by inhibiting inflammation and/or simultaneously protect neurons and oligodendrocytes from damage, could thus be effective therapies for multiple sclerosis.

Keywords: glutamate receptor antagonist; MAdCAM-1 antibody; encephalomyelitis; cell adhesion

Abbreviations: AMPA = α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate; CNPase = 2′,3′-cyclic nucleotide 3′-phosphodiesterase; DAB = diaminobenzidine; EAE = experimental autoimmune encephalomyelitis; ELISA = enzyme-linked immunosorbent assay; GFAP = glial fibrillary acidic protein; GluR = glutamate receptor; GPE = glycine–proline–glutamic acid (GPE; N-terminal tripeptide of insulin-like growth factor). Remarkably, administration at an advanced stage of unremitting EAE of either a combination of NBQX and GPE, or preferably all three latter reagents, resulted in amelioration of disease and repair of the CNS, as assessed by increased oligodendrocyte survival and remyelination, and corresponding decreased paralysis, inflammation, CNS apoptosis and axonal damage. Each treatment reduced the expression of nitric oxide and a large panel of proinflammatory and immunoregulatory cytokines, in particular IL-6 which plays a critical role in mediating EAE. Mice displayed discernible improvements in all physical features examined. Disease was suppressed for 5 weeks, but relapsed when treatment was suspended, suggesting treatment must be maintained to be effective. The above approaches, which allow CNS repair by inhibiting inflammation and/or simultaneously protect neurons and oligodendrocytes from damage, could thus be effective therapies for multiple sclerosis.

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Introduction

Multiple sclerosis is a disease in which the immune system attacks the white matter of the brain and spinal cord, leading to disability and/or paralysis (Martin and McFarland, 1995; Wingerchuk et al., 2001). Myelin, oligodendrocytes and neurons are lost due to an inflammatory attack by leukocytes that infiltrate the CNS and release cytotoxic cytokines, anti-CNS autoantibodies and large amounts of the excitatory neurotransmitter glutamate. The pathways by which encephalitogenic T cells access the CNS have not been fully delineated, but clearly involve integrin cell adhesion molecules, which mediate lymphocyte traffic. A role for α4 and β7 integrins and their ligand, mucosal addressin cell adhesion molecule-1 (MAdCAM-1), in the establishment of experimental autoimmune encephalomyelitis (EAE) was demonstrated by in vivo administration of anti-α4 integrin (Yednock et al., 1992; Baron et al., 1993; Kanwar et al., 2000b), anti-β7 integrin (Kanwar et al., 2000b) and anti-MAdCAM-1 (Kanwar et al., 2000a) monoclonal antibodies (mAbs), which diminished the paralysis associated with EAE when administered early in the disease process. In contrast, anti-vascular addressin therapy was not effective when administered at a late stage of disease (Kanwar et al., 2000a). A humanized anti-integrin α4 subunit antibody reduced the rate of new MRI lesion formation in multiple sclerosis, but did not abolish it altogether (Tubridy et al., 1999).

Integrin-mediated entry of leukocytes into the CNS leads to markedly elevated concentrations of glutamate, a major excitatory amino acid neurotransmitter (Stover et al., 1997). Glutamate receptors of the AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate) and kainate class are expressed on both neurons and oligodendrocytes, whereas receptors of the N-methyl-D-aspartate (NMDA) class are found on neurons. Low concentrations of glutamate, AMPA and kainate are toxic to oligodendrocytes and neurons in vitro (Ikonomidou et al., 1996; McDonald et al., 1998). The AMPA/kainate receptor antagonist 2,3-dihydroxy-6-nitro-7-sulfamoylbenzo(f)quinoxaline (NBQX) (Sheardown et al., 1990) inhibits the excitotoxic effects of glutamate on oligodendrocytes and has been found to ameliorate the pathogenesis of relapsing-remitting EAE in myelin basic protein (MBP)-induced Lewis rat and SJL/J mice models of multiple sclerosis (Pitt et al., 2000; Smith et al., 2000) when administered at an early stage of disease, but could not block the disease process permanently.

Insulin-like growth factor-1 (IGF-1) is another neuroprotector, which reduces demyelination, lesion size and numbers, and slightly reduces the severity of EAE (Liu et al., 1997; Lovett-Racke et al., 1998). An N-terminal tripeptide fragment of IGF-1, glycine-proline-glutamic acid (GPE), was first identified as a novel neuroactive peptide by Sara et al. (1989). It retains some of the neuroprotective effects of IGF-1, as demonstrated in animal models of hypoxic-ischaemic brain injury (Guan et al., 1999) and Huntington’s disease (Alexi et al., 1999). GPE was shown to exhibit cross-receptor activity in that it inhibited glutamate binding to the NMDA receptor and prevented neuronal death in the hippocampus injured by NMDA (Saura et al., 1999). Amantadine was found to reduce the relapse rate in 53 patients with multiple sclerosis in a double-blind multicentre study, but the results would need to be repeated with a larger patient cohort to confirm the efficacy (Plaut, 1987). Other NMDA antagonists such as memantine (Wallstrom, et al., 1996) and MK801 (Bolton and Paul, 1997) abrogate neurological deficits, but not CNS inflammation in EAE in rats.

In this study, we investigated whether advanced stage EAE, which to date has remained intractable to treatment, can be treated by blockade of the inflammatory response with an anti-MAdCAM-1 mAb, coupled with protection of oligodendrocytes and neurons against glutamate damage with a combination of the AMPA/kainate antagonist NBQX and the neuroprotector GPE.

Methods

Induction of unremitting EAE

C57BL/6 mice (8–10 weeks old) were injected subcutaneously in one flank with 300 μg of myelin oligodendrocyte glycoprotein 35–55 (MOG35–55) peptide (MEVGWYRSPFSRVHLYRNGK), synthesized by Chiron Technologies Ltd, Clayton, Australia and emulsified in complete Freund’s adjuvant (CFA) containing 500 μg of Mycobacterium tuberculosis H37Ra (DIFCO Laboratories, Detroit, USA). They also received 500 ng of pertussis toxin (List Biological Laboratories, CA, USA) in 200 μl phosphate-buffered saline (PBS) injected intravenously via the tail vein, followed 48 h later by a second dose. A second injection of MOG peptide was given in the absence of pertussis toxin one week later in the opposite flank, according to a previously accepted protocol (Mendel et al., 1995; Kanwar et al., 2000a).

Treatment of EAE

The incidence of disease using the above induction protocol varied from 50 to 100%. Only mice that developed EAE were studied. Antibody (500 μg) was administered into the tail vein (70% of mAb) and intraperitoneally (30% of mAb) at 10 mg/kg; whereas for the neuroprotectors (30 μg GPE and 6 mg NBQX), 50% of reagent was given intraperitoneally and 50% intravenously. ‘Mock-treated’ mice received either rat IgG at 10 mg/kg (Sigma Co., St Louis, MO, USA) or PBS. Unless otherwise indicated, five mice were included in each treatment group for clinical scoring and an additional five mice were included for analysis of neuropathology. The rat hybridoma MECA-367 (rat IgG2a) (Berlin et al., 1993), which secretes a mAb against mouse MAdCAM-1 was provided by Dr Eugene Butcher, Stanford University, Stanford, CA, and also obtained from the American Type Culture Collection, Rockville, MD. NBQX was obtained from ICN Biomedicals, Costa Mesa, CA, USA, and GPE from Bachem, Torrance, CA, USA.

Scoring of disease and disability

Mice were monitored daily and neurological impairment scored according to the following scale: 0 = no clinical signs of EAE; 1 = limp tail; 2 = partial hind limb paralysis; 3 = complete hind limb...
paralysis; 4 = complete hind limb and partial fore limb paralysis; 5 = paralysis extending to diaphragm; 6 = hind and fore limb paralysis; 7 = death due to EAE. Paralysis extending to the diaphragm was denoted as difficulty in remaining upright. Animals were cared for according to the guidelines of the University of Auckland Animal Ethics Committee. The daily mean clinical score for each group is the mean disease score of at least five mice, and each experiment was repeated at least once, unless otherwise indicated.

**Immunohistochemical analysis of neuropathology**

Transverse 10 μm sections made through the spinal cord were stained with haematoxylin and eosin, and examined by light microscopy. Data presented in Figs 4–7 are from groups of five mice at day 75, whose clinical scores are shown in Figs 5–7. Data in Fig. 4 and Tables 2 and 3 are derived from a separate experiment involving 10 mice, where two mice were selected for measuring the apoptotic index and inflammation score, at each time point. The disease scores for these latter mice were comparable to those shown in Fig. 3. One-μm epoxy sections of cerebrum, cerebellum/brainstem, cervical, thoracic, lumbar and sacral spinal cord, and spinal nerve roots were stained with toluidine blue (Sigma) and examined by light microscopy. A score from 0 to 5 was determined for inflammation, according to established criteria (Moore et al., 1984; Cross et al., 1994).

An antibody to exon 2 of MBP was raised in a rabbit by coupling a mouse MBP exon 2 peptide (DSHTRTTHGSLPQKSQHG-RTOQDENPVHFFKNCG) to the carrier protein thyroglobulin. Frozen sections from lumbar spinal cord (10 μm) were immunostained for oligodenrocyte content with an antibody against 2′,3′-cyclic nucleotide 3′-phosphodiesterase (CNPase, Sigma; diluted 1:100); for axonal damage with an antibody against non-phosphorylated neurofilament-H (SM1-32; Sternerberger Monoclonals Incorporated, Lutherville, MA, USA; diluted 1:1000), and for glutamate receptor subunit 2 (Glur2) content using a mouse mAb obtained from Zymed Laboratories Inc., Carlton, San Francisco, CA, USA (diluted 1:1000). Antibody staining was visualized with an avidin:biotinylated enzyme complex (Vector Laboratories, Burlingame, CA, USA).

**Measurement of cell type specific apoptosis**

Sections were incubated with fluorescein isothiocyanate (FITC) linked TUNEL (terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate-digoxigenin nick end labelling) (Boehringer-Mannheim, Mannheim, Germany), and subsequently overnight at 4°C with cell-type-specific and anti-mouse caspase-3/CPP32 mAbs, followed by detection using tetramethyl rhodamine isothiocyanate (TRITC) conjugated goat anti-mouse immunoglobulin G (IgG) or anti-rat IgG, or goat anti-rabbit IgG (1:100; Sigma). Slides were examined under a Nikon Eclipse E600 fluorescence microscope fitted with a DXM1200 digital camera. Analysis of coded sections was performed by an observer blinded to the treatment, who rated 10 visual fields (1.6 mm²) per section of spinal cord at 250× magnification from five sections obtained at different levels of the spinal cord per animal. Apoptotic oligodenrocytes and neurons were determined in 0.01 mm² fields at 1000× magnification. Sections were also examined on a Leica TCS 4D confocal microscope (Leica Microsystems, GmbH, Heidelberg, Germany) (Kanwar et al., 2001a, b) and compiled from sets of three to five consecutive single optical sections using Leica Scanware™ 4.2A software (Leica Lasertechnik, GmbH, Heidelberg, Germany). Alternatively, tissue sections were immunostained by the ABC (avidin-biotinylated enzyme complex) method using diaminobenzidine (DAB) (Vector Laboratories, Inc., Burlingame, CA, USA), then briefly counterstained with haematoxylin.

**Western blot analysis, TUNEL, ELISA, RNase protection and anti-MOG35-55 antibody assays**

More detailed methods including methods for western blot analysis, TUNEL, enzyme-linked immunosorbent assay (ELISA), RNase protection assays (RPAs) and anti-MOG35-55 antibody assays are provided as online supplementary material.

**Results**

**Blockade of MAdCAM-1 prevents the development of an unremitting form of EAE**

We induced an unremitting form of EAE (Mendel et al., 1995; Kanwar et al., 2000a) by immunizing C57BL/6 mice subcutaneously with MOG35-55 peptide emulsified in CFA containing Mycobacterium tuberculosis H37Ra), and subsequently administering pertussis toxin. The first signs of clinical EAE ensued between days 28 to 35, with a gradual decrease in the average body weight of mice from 23–24 g to 11 g within 2 weeks (Figs 1, 2 and 3). There was progressive development of tail and hind limb paralysis from day 35, which reached a peak at days 42 to 45 (Figs 2 and 3). The delayed start of disease and high clinical score, which persisted at the same level for several months, are characteristic of MOG-induced EAE in the C57BL/6 strain of mice (Mendel et al., 1995). In confirmation of a role for the vascular addressin MAdCAM-1, three intravenous and intraperitoneal injections of anti-MAdCAM-1 mAb given on days 35, 37 and 39 (following injection of autoantigen) completely prevented the induction of EAE such that no overt clinical symptoms could be observed for 57 days after suspension of antibody treatment (Fig. 2).

**Neuroprotectors suppress but do not prevent the development of EAE**

NBQX given daily for 6 days (days 35–40) following injection of autoantigen suppressed MOG35-55-induced EAE for at least 14 days following suspension of treatment (mean disease score 0.7 ± 0.4 compared with 4.1 ± 1.5 for control rat IgG at day 54) but, unlike the results achieved with anti-MAdCAM-1 mAb treatment, disease severity gradually
increased 3 days following suspension of treatment (Fig. 2). GPE also suppressed EAE for at least 14 days following suspension of treatment (mean disease score 0.7 ± 0.4 compared with 4.1 ± 1.5 for control rat IgG at day 54) when given as a short-term treatment for 6 days from day 35 (Fig. 2). However, thereafter disease severity gradually increased as with NBQX. In summary, each treatment form was able to inhibit the progression of EAE when administered early in the course of disease, where the effectiveness of treatment was anti-MAdCAM-1 mAb > NBQX or GPE. As monotherapies, only anti-MAdCAM-1 mAb treatment was able to completely prevent the development of early stage EAE. Surprisingly, combined treatment with GPE and NBQX had a synergistic effect, leading to sustained suppression of disease symptoms for at least 55 days the animals were monitored following suspension of treatment (Fig. 2). Further, simultaneous administration of anti-MAdCAM-1 mAb, NBQX and GPE also delivered sustained protection (mean disease score reduced from 3.5 ± 0.5 at day 59 (before treatment) to 1.2 ± 0.7 at day 69 (3 days after suspension of treatment)) for 24 days following suspension of treatment. The triple combination of anti-MAdCAM-1 mAb, NBQX and GPE appeared to be synergistic as the disease was suppressed [mean disease score reduced from 3.5 ± 0.7 at day 59 (before treatment) to 1.2 ± 0.7 at day 69 (3 days after suspension of treatment)] for 24 days following suspension of treatment. Further, simultaneous administration of anti-MAdCAM-1 mAb, NBQX and GPE also delivered sustained protection [mean disease score reduced from 3.5 ± 0.5 at day 59 (before treatment)].

Simultaneous blockade of integrin and GluRs ameliorates advanced EAE

We next tested the above mono- and combination therapies for their ability to combat EAE at an advanced stage in which mice had suffered from complete hind limb paralysis for 3 weeks. Administration of three injections (beginning on day 51 after injection of autoantigen) of high dose anti-MAdCAM-1 mAb when mice had been paralysed for at least 10 days caused initial remission of disease, which was quickly followed by a gradual and complete relapse after suspension of treatment (Fig. 3A). NBQX and GPE administered for 7 days as monotherapies from day 60 caused only temporary remission and, by day 77, the disease had relapsed (Fig. 3A). Surprisingly, the combination of NBQX with GPE appeared to be synergistic as the disease was suppressed [mean disease score reduced from 3.5 ± 0.7 at day 59 (before treatment) to 1.2 ± 0.7 at day 69 (3 days after suspension of treatment)] for 24 days following suspension of treatment. Further, simultaneous administration of anti-MAdCAM-1 mAb, NBQX and GPE delivered sustained protection [mean disease score reduced from 3.5 ± 0.5 at day 59 (before treatment)].
treatment) to 0.8 ± 0.2 at day 69 (3 days after suspension of treatment) (Fig. 3A). In summary, there was strong evidence of a difference in the shape of the therapeutic effect over time in the treatment groups \[F(5,503) = 5.7, P < 0.0001\] with the results obtained with the rat IgG control differing from those obtained with the therapeutic agents \[F(1,503) = 25.6, P < 0.0001\], combined treatments differing from monotherapies \[F(1,503) = 20.7, P < 0.0001\], and anti-MAdCAM-1 mAb monotherapy differing from GPE and NBQX monotherapies \[F(1,503) = 18.3, P < 0.0001\]. Neither the GPE and NBQX monotherapies nor the 2 combinations (GPE+NBQX and GPE+NBQX+anti-MAdCAM-1 mAb) could be shown to differ from one another \[F(1,503) = 1.2, P = 0.28\] and \[F(1,503) = 0.14, P = 0.71\], respectively.

In a repeat experiment, the various treatment regimes were administered for 18 days from day 60 (Fig. 3B). Administration of eight injections (beginning on day 60 after injection of autoantigen) of high dose anti-MAdCAM-1 mAb when mice had been paralysed for at least 20 days caused only slight remission of disease, which again was quickly followed by a gradual and complete relapse after suspension of treatment (Fig. 3B). NBQX and GPE administered as monotherapies for 18 days from day 60 again moderately ameliorated disease (NBQX, mean clinical score 1.8 ± 0.6 at day 69; GPE mean clinical score 1.8 ± 0.8 at day 69) until days 98 and 77, respectively (Fig. 3B). However, the triple [NBQX + GPE + anti-MAdCAM-1 mAb (mean clinical score 0.8 ± 0.6 at day 69)] and double [NBQX + GPE (mean clinical score 1.3 ± 0.4 at day 69)] treatments were again more effective at ameliorating disease but, as with the other treatments, disease eventually relapsed. There was strong evidence of a difference in the shape of the therapeutic effect over time in the groups \[F(6,615) = 5.7, P < 0.0001\], with control groups differing from the active therapeutic treatments \[F(6,615) = 21.5, P < 0.0001\], combined treatments differing from monotherapies \[F(6,615) = 4.6, P = 0.03\], and anti-MAdCAM mAb treatment differing from GPE and NBQX monotherapies \[F(6,615) = 6.3, P = 0.01\]. No difference could be shown between the two combinational treatments, or between the GPE and NBQX monotherapies \[F(6,615) = 0.18, P = 0.67\] and \[F(1,615) = 0.02, P = 0.90\], respectively. In summary, NBQX and GPE monotherapies that prevent glutamate excitotoxicity appear more effective than anti-cell adhesion reagents at treating advanced stage demyelinating disease, whereas anti-cell adhesion reagents are more effective at preventing early disease. The disease returned in all cases following suspension of treatment, suggesting that therapy must be maintained to prevent relapse. No mice died in any of the different treatments, suggesting that the treatment protocols are safe and effective at the doses employed.

**Mice experience a weight gain following therapy**

Mice experienced a weight gain following treatment, which correlated with the efficacy of the particular treatment regime, where the weight of mice receiving the triple treatment (mean average body weight 21.6 ± 6.9 compared with 13.2 ± 4.3 for the rat IgG control group at day 119) or double treatment (GPE + NBQX, mean average body weight 20.8 ± 4.8) increased by >50% (Fig. 1).

**Disability scores confirm the relative efficacies of the different treatment regimes**

We sought to determine how signs of disability, scored using a previously developed disability scale (Villoslada et al., 2000) correlated with each treatment regime. Disability
scores correlated with the clinical scores of paralysis (Table 1). Treated animals displayed discernible improvements in alertness, spontaneous mobility, tone, motor function (grip), sensory function, shiny hair/skin firmness and decreased tremor. The total scores at day 75 were 361 (P < 0.001) for NBQX + GPE + anti-MAdCAM-1 mAb, 1063 (P < 0.001) for NBQX + GPE, 1963 (P < 0.001) for anti-MAdCAM-1 mAb, 1062 (P < 0.001) for GPE, and 3064 (P > 0.05) for NBQX, compared with 3563 for rat IgG treated control mice. The relatively high score for NBQX was consistent with a short relapse for NBQX treated mice at this time point (Fig. 3B), whereas scores preceding and after the relapse were similar to those achieved with GPE. There was strong evidence of a difference (P < 0.001) in the disability scores in response to the triple (NBQX + GPE + anti-MAdCAM-1 mAb) versus the double (NBQX + GPE) treatment, and each of the monotherapies. The double treatment was statistically different from anti-MAdCAM-1 mAb blockade (P < 0.001), but not from GPE treatment alone (P > 0.05).

**Simultaneous blockade of integrin and GluRs reduces CNS apoptosis and axonal damage in advanced EAE**

A neuropathological evaluation of damage to the brain and spinal cord was carried out to determine the molecular basis for the therapeutic efficacy of the combination treatment regimes. TUNEL analysis of brain and spinal cord sections from EAE mice revealed extensive apoptosis, which peaked...
Table 1 Disability status scale

<table>
<thead>
<tr>
<th>Disability function</th>
<th>Maximal disability scores (mean values)</th>
<th>Rat IgG</th>
<th>GPE</th>
<th>NBQX</th>
<th>GPE + NBQX</th>
<th>MAdCAM-1</th>
<th>GPE + NBQX + MAdCAM-1</th>
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<td></td>
<td></td>
<td>55</td>
<td>60</td>
<td>65</td>
<td>70</td>
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<td>1</td>
<td>1 2 1 1 1 1</td>
<td>2 2 1 1 1 1</td>
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<tr>
<td>2. Spontaneous mobility</td>
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<td>3 3 2 3 3 2</td>
<td>3 3 2 3 3 2</td>
<td>3 3 2 3 3 2</td>
<td>3 3 2 1 0 2</td>
<td>3 3 0 0 1 1</td>
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<td>3. Tremor</td>
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<td>4. Tone (b)</td>
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<td>12 12 8 4 4 4</td>
<td>12 12 8 4 4 4</td>
<td>12 12 8 4 4 4</td>
<td>12 12 8 4 4 4</td>
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<td>5. Motor (grip) (b)</td>
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<td>12 12 4 4 4 0</td>
<td>12 12 4 4 4 0</td>
<td>12 12 4 4 4 0</td>
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<td>Light touch</td>
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<td>8. Vision (including pupillary reflex)</td>
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<td>9. Bladder function</td>
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<td>10. Other signs (shiny hair and loose skin)</td>
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<td>Total disability score</td>
<td></td>
<td>51 53 43 51 35 42</td>
<td>52 52 15 13 10 10 17</td>
<td>53 53 15 13 30 23</td>
<td>52 52 15 12 10 17</td>
<td>52 52 15 7 19 19 17</td>
<td>48 52 0 5 3 3 12</td>
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</table>

\(a\)The total score is derived by adding the scores for each disability from five mice. Data represent mean values where SD values are provided only for the total disability scores.

\(b\)Scored in each limb. \(c\)Scored only if tactile not present. *Indicates significantly different at day 75 from the rat IgG control, using ANOVA followed by a Bonferroni and Dunnett test for multiple comparisons \((P < 0.001)\). \(i\)Indicates significantly different at day 75 from the rat IgG control, and from monotherapy and double GPE plus NBQX therapy, using ANOVA followed by a Bonferroni and Dunnett test for multiple comparisons \((P < 0.001)\).
at the height of disease severity (day 45) and then subsequently declined to a sustained plateau (Fig. 4A). Sections taken from NBQX + GPE + anti-MAdCAM-1 mAb, and anti-MAdCAM-1 mAb treated mice at day 75 had substantial reductions (60%; $P < 0.05$) in the numbers of apoptotic cells compared with mice that had been mock treated with either rat IgG or PBS treated control mice at day 75 were performed using ANOVA followed by the Bonferroni–Dunnett test for multiple comparisons. Differences in the AI were significant ($P < 0.05$, as indicated by asterisk). For clarity, the time interval on the x-axis is not drawn to scale. (B) Double immunofluorescence staining with fluorescein-linked TUNEL reveals apoptotic neurons and oligodendrocytes. Illustrated are confocal images of serial spinal cord sections from EAE mice at day 75. TUNEL positive neurons (top six panels) were stained with anti-caspase 3 (A) and anti-neuron-specific protein (NeuN) mAbs (B), and the images merged. TUNEL positive oligodendrocytes (bottom six panels) were stained with an anti-caspase 3 (C) and anti-CNPase mAb (D) and the images merged (middle three panels). Overlapping stains produce yellow-coloured cells. Scale bars represent 20 µm.

Fig. 4 Apoptosis in the CNS as assessed by TUNEL staining and immunohistochemistry. (A) Serial sections of the brain and spinal cord were TUNEL stained to detect cells undergoing apoptosis. The percentage of apoptotic cells was assessed in 10 randomly selected fields to provide the apoptotic index. Arrows refer to antibody administration, whereas the dotted line refers to administration of neuroprotectors. Data are expressed as a percentage of apoptotic index (AI) and represent mean ± SD ($n = 5$). Comparisons between MAdCAM-1 mAb and GPE+NBQX+MAdCAM-1 mAb treated mice with rat IgG or PBS treated control mice at day 75 were performed using ANOVA followed by the Bonferroni–Dunnett test for multiple comparisons. Differences in the AI were significant ($P < 0.05$, as indicated by asterisk). For clarity, the time interval on the x-axis is not drawn to scale. (B) Double immunofluorescence staining with fluorescein-linked TUNEL reveals apoptotic neurons and oligodendrocytes. Illustrated are confocal images of serial spinal cord sections from EAE mice at day 75. TUNEL positive neurons (top six panels) were stained with anti-caspase 3 (A) and anti-neuron-specific protein (NeuN) mAbs (B), and the images merged. TUNEL positive oligodendrocytes (bottom six panels) were stained with an anti-caspase 3 (C) and anti-CNPase mAb (D) and the images merged (middle three panels). Overlapping stains produce yellow-coloured cells. Scale bars represent 20 µm.
PBS or rat IgG. In contrast, sections taken from NBQX + GPE, NBQX, and GPE treated mice showed lesser reductions (~30%; \( P > 0.05 \)) in the numbers of apoptotic cells (refer to apoptotic index, Fig. 4A).

Double immunofluorescence staining for TUNEL and cellular markers was able to determine that all cell types examined in the CNS of EAE mice at day 75 were subject to apoptosis (Table 2). Thus, 33–37% of oligodendrocytes, 16% of neurons, 17–27% of CD11+ T cells, and 17–20% of Mac-1+ macrophages were apoptotic. Apoptotic neurons and oligodendrocytes expressed the apoptosis effector caspase 3 (Fig. 4B). The triple treatment was very effective in reducing the apoptosis of oligodendrocytes and neurons, by 83%, and 89%, respectively, with respect to PBS control (\( P < 0.001 \)) (Table 2). Anti-MAdCAM-1 mAb and GPE + NBQX effectively inhibited the apoptosis of oligodendrocytes by 62% and 42%, respectively, whereas GPE and NBQX individually were only slightly effective. In contrast, GPE and NBQX effectively inhibited the apoptosis of neurons by 56% and 45%, respectively, and together caused 70% inhibition. Anti-MAdCAM-1 mAb was equally effective, causing 69% inhibition. As regards T cells, assessment of the affects of the agents on T cell apoptosis is complicated by the fact that the triple, double and anti-MAdCAM-1 mAb treatments

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**Fig. 5** Assessment of axonal damage by measuring levels of abnormally dephosphorylated neurofilament-H. (A) Axonal dystrophy in transverse spinal cord sections stained for dephosphorylated neurofilament-H using the SM1-32 mAb. Sections were prepared from mice treated with the various reagents as indicated. Magnification: 40×. (B) Densitometric analysis of western blots of dephosphorylated neurofilament-H in spinal cord homogenates. Western blots containing pooled spinal cord homogenates from five representative mice per treatment group per lane were stained with the SM1-32 mAb (top panel). Clinical scores at day 75 are as indicated. The relative amounts of dephosphorylated neurofilament-H in each lane were recorded by densitometry. The order of the homogenates shown in the histograph is the same for the western blot. (C) Numbers of damaged axonal cells staining for dephosphorylated neurofilament-H. Data represent the means ± SD. Comparisons between mice treated with therapeutic agents and those treated with control rat IgG at day 75 were performed by ANOVA followed by the Dunnett test for multiple comparisons and statistical significance indicated as *\( P < 0.05 \), **\( P < 0.001 \).
Table 2 Apoptosis of different brain and inflammatory cell types in the CNS

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Oligodendrocytes/mm² (mean± SD)</th>
<th>Neurons/mm² (mean± SD)</th>
<th>T cells/mm² (mean± SD)</th>
<th>Macrophages/mm² (mean± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CNPase positive cells</td>
<td>CNPase/TUNEL&lt;sup&gt;a&lt;/sup&gt; positive cells</td>
<td>NeuN positive cells</td>
<td>NeuN/TUNEL&lt;sup&gt;a&lt;/sup&gt; positive cells</td>
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<tr>
<td>Normal</td>
<td>85 ± 12</td>
<td>-</td>
<td>110 ± 12</td>
<td>-</td>
</tr>
<tr>
<td>PBS</td>
<td>70 ± 10</td>
<td>26 ± 6 (37)</td>
<td>100 ± 15</td>
<td>16 ± 6 (16)</td>
</tr>
<tr>
<td>GPE + NBQX + MAdCAM</td>
<td>78 ± 9</td>
<td>5 ± 3** (6.4)</td>
<td>112 ± 13</td>
<td>2 ± 2** (1.8)</td>
</tr>
<tr>
<td>MAdCAM</td>
<td>72 ± 8</td>
<td>10 ± 5 (14)</td>
<td>102 ± 12</td>
<td>5 ± 3 (4.9)</td>
</tr>
<tr>
<td>GPE + NBQX</td>
<td>68 ± 8</td>
<td>15 ± 6 (22)</td>
<td>105 ± 16</td>
<td>5 ± 2 (4.8)</td>
</tr>
<tr>
<td>GPE</td>
<td>70 ± 12</td>
<td>20 ± 8 (29)</td>
<td>116 ± 15</td>
<td>8 ± 3 (6.9)</td>
</tr>
<tr>
<td>NBQX</td>
<td>64 ± 9</td>
<td>22 ± 6 (34)</td>
<td>113 ± 18</td>
<td>10 ± 4 (8.8)</td>
</tr>
<tr>
<td>Rat IgG</td>
<td>75 ± 12</td>
<td>25 ± 5 (33)</td>
<td>115 ± 14</td>
<td>18 ± 7 (16)</td>
</tr>
</tbody>
</table>

<sup>a</sup>To confirm apoptosis, we determined whether TUNEL positive cells also expressed caspase-3. *Indicates anti-MAdCAM-1 mAb therapy is significantly (P < 0.05) different from GPE and NBQX monotherapies, and combination GPE+NBQX therapy. **Indicates significantly (P < 0.001) different at day 75 from the rat IgG/PBS control, using ANOVA followed by a Bonferroni and Dunnett test for multiple comparisons. Also indicates significantly (P < 0.001) different at day 75 from GPE and NBQX monotherapies, combination GPE+NBQX therapy, and anti-MAdCAM-1 mAb therapy using ANOVA followed by a Bonferroni and Dunnett test for multiple comparisons. Numbers in brackets refer to percentage of apoptotic cells. Data represent mean values ± SD.
reduced the overall numbers of T cells in the CNS by 55%, 40% and 47%, respectively. In the case of the triple treatment, those T cells able to enter the CNS were less apoptotic (apoptosis reduced by 55%), whereas the other therapies increased T cell apoptosis by 200–300%. The numbers of apoptotic Mac-1+ macrophages were too small to make reliable comparisons. The triple treatment significantly \((P < 0.001)\) reduced the apoptosis of oligodendrocytes, neurons and T cells compared with all other therapies.

Axonal damage is a critical feature of multiple sclerosis lesions. Dephosphorylated heavy chain of neurofilament-H is a quantitative molecular marker of demyelinated and dystrophic axons (Trapp et al., 1998). Immunohistochemistry of spinal cord sections (Fig. 5A and C) and western blot analysis of spinal cord homogenates (Fig. 5B).

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**Fig. 6 Survival of oligodendrocytes and remyelination.** (A) Transverse sections of spinal cord were stained with an antibody against CNPase to visualize oligodendrocytes within dorsal columns. Sections from a representative mouse treated with the combination of anti-MAdCAM-1 mAb + NBQX + GPE were compared with those from a normal undiseased mouse. The upper panels are at magnification \(20\times\) and middle panels at \(100\times\). An anti-CNPase mAb stained section from a rat IgG-treated mouse (lowest panel as indicated, magnification \(100\times\)) is included as a control. (B) Enumeration of oligodendrocytes, and assessment of CNPase activity in spinal cord sections. Western blots containing pooled spinal cord homogenates from five representative mice per treatment group per lane were stained with an anti-CNPase mAb \(\text{(top panel)}\). Clinical scores at day 75 are as indicated. The histogram shows enumeration of oligodendrocytes in dorsal columns of transverse sections of spinal cord stained with an antibody against CNPase. Each bar represents the average number of oligodendrocytes from two representative mice, with 5–10 sections analysed per mouse. Data represent means ± SD. Comparisons between mice treated with therapeutic agents and those treated with control rat IgG at day 75 were performed by ANOVA followed by the Bonferroni–Dunnett test for multiple comparisons and statistical significance indicated as \(*P < 0.05, **P < 0.001\).

(C) Assessment of remyelination by staining for exon 2 of MBP. Transverse sections of the spinal cord, as indicated, were immunostained with an antibody against exon 2 of MBP to visualize areas undergoing remyelination. (D) Assessment of remyelination by western blot analysis of the expression of exon 2 of MBP in spinal cord homogenates. Western blots containing pooled spinal cord homogenates from five representative mice per treatment group per lane were stained with an anti-exon 2 MBP antibody \(\text{(top panel)}\). The arrow indicates the position of the 18 kDa MBP band. Clinical scores at day 75 are as indicated. The relative amounts of exon 2 of MBP in each lane were recorded by densitometry. The order of the homogenates shown in the histogram is the same for the western blot.
prepared at day 75 revealed that the spinal cords of mock-treated (rat IgG, PBS) EAE mice (disease score 4.5) displayed a large increase of abnormally dephosphorylated neurofilament-H (Fig. 5A and B) and contained increased numbers of damaged axonal cells (Fig. 5C), whereas these variables were almost undetectable in normal undiseased mice. The levels of dephosphorylated neurofilament-H, and numbers of damaged axonal cells expressing dephosphorylated neurofilament-H after treatment largely correlated with the efficacy of the particular treatment regime. For example, the average relative densities of neurofilament-H in day 75 spinal cord homogenates resolved by western blot analysis were 1.2 ± 0.4 for NBQX + GPE + anti-MAdCAM-1 mAb, 2.2 ± 0.4 for NBQX + GPE, 2.3 ± 0.7 for anti-MAdCAM-1 mAb, 3.2 ± 0.6 for GPE and 3.5 ± 0.6 for NBQX compared with 6.1 ± 2.1 for rat IgG and 6.2 ± 2.1 for PBS-treated control mice (Fig. 5B). GPE and NBQX administered individually almost halved the numbers of axonal cells expressing dephosphorylated neurofilament-H at day 75 and, in combination, further reduced the number by 30% (P < 0.05) (Fig. 5B), indicating that glutamate excitotoxicity is a key feature contributing to axonal damage in MOG-induced EAE. When axonal damage was assessed, GPE (P < 0.05) and anti-MAdCAM-1 mAb treatment (P < 0.05), but not NBQX (P > 0.05), caused a statistically significant reduction in damage. In accord, there was no statistical difference (P > 0.05) between GPE alone and the combination of GPE and NBQX (Fig. 5C). Thus, in this instance, the levels of the dephosphorylated heavy chain of neurofilament-H did not correlate exactly with the level of axonal damage. There was no significant difference between anti-MAdCAM-1 mAb treatment and the combination of GPE + NBQX at reducing axonal damage (P > 0.05) (Fig. 5B and C), as also evidenced by a reduction in the overall apoptotic index (Fig. 4), despite the fact that anti-MAdCAM-1 mAb was therapeutically less effective in reducing the overall clinical score. The triple treatment (NBQX + GPE + anti-MAdCAM-1 mAb) was more effective than the double treatment (NBQX + GPE) (P < 0.05), and each of the monotherapies (P < 0.05). In summary, the anti-inflammatory and neuroprotective agents described herein act in concert to reduce the degree of axonal damage, as reflected in the attenuated neuropathology, and clinical symptoms of disease.

Simultaneous blockade of integrin and GluRs prevents loss of oligodendrocytes and facilitates repair of the CNS

Oligodendrocyte numbers were enumerated to evaluate the effects of the different treatment regimes on the loss of oligodendrocytes. Sections prepared at day 75 were stained with an antibody against the oligodendrocyte marker CNPase (Fig. 6A) and oligodendrocytes within the dorsal columns of 5–10 transverse sections were counted (Fig. 6B). Preservation of oligodendrocyte numbers correlated with the efficacy of the different treatment regimes. For example, the numbers of oligodendrocytes were 120 ± 18 (P < 0.001) for NBQX + GPE + anti-MAdCAM-1 mAb, 110 ± 15 (P < 0.05) for NBQX + GPE, 104 ± 15 (P > 0.05) for NBQX, 102 ± 17 (P > 0.05) for anti-MAdCAM-1 mAb, 93 ± 14 (P > 0.05) for GPE versus 74 ± 10 for control PBS-treated mice. The results of the triple and double treatments were statistically different from each other (P < 0.05), and the triple treatment was significantly different (P < 0.05) from the monotherapies. The double treatment was statistically different from each of the monotherapies (P < 0.05), except for NBQX (P > 0.05). These results were confirmed by western blot analysis to quantitate the amount of CNPase in spinal
cord homogenates (Fig. 6B). We also investigated whether repair due to remyelination correlated with reduced loss of oligodendrocytes. Spinal cord sections at day 75 were stained with an antibody against exon 2 of mouse MBP. Exon 2 containing MBP transcripts (Jordan et al., 1990; Nagasato et al., 1997) and protein (Kruger et al., 1999) are expressed de novo during remyelination in rodents and humans. Distinct patches of remyelinated tissue in both the grey and white matter of the spinal cord stained with the anti-exon 2 MBP antibody, where the number of patches correlated with the efficacy of each treatment regime (Fig. 6C; data is provided for the triple treatment only). Similar results were achieved by western blot analysis of exon 2 MBP content in spinal cord homogenates, where the anti-exon 2 MBP antibody detected a single MBP band of 18 kDa (Fig. 6D). The levels of expression of exon 2 of MBP correlated with the efficacies of each treatment regime. Remyelination was increased 4–5 fold by the double and triple treatment regimes.

**Combinational therapy suppresses the up-regulation of ionotropic GluRs**

Levels of the GluR subunits 1 and 2 (GluR1 and 2) increase in the rat spinal cord after inflammation (Zhou et al., 2001), suggesting their up-regulation is an indicator of disease. As shown in Fig. 7A, large numbers of cell bodies and dendrites within the grey matter (dorsal and ventral horns, and intermediate zone) stained for GluR2. Differential staining

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**Fig. 8** Blockade of integrin and glutamate receptors decreases the expression of proinflammatory and anti-inflammatory cytokines.

(A) Multiprobe RPA analysis of cytokine mRNA expression in the CNS was performed (see Methods) using the mCK1b, mCK2b and mCK3b multiprobe template sets. The gel was imaged and the ratio of the protected bands normalized to the mean sum of the protected bands for mL32 plus GAPDH (glyceraldehyde-3-phosphate dehydrogenase). Data were quantified and are presented as the mean ± SD from two different experiments. (B) ELISA of cytokine and nitrite levels. Results were expressed as the mean concentration ± SD for IFN-γ, TNF-α, IL-6, IL-10, IL-12, IL-5 (pg/ml) and for nitrite (µM), as shown. Data represent means ± SD. Comparisons between mice treated with therapeutic agents and those treated with control rat IgG at day 75 were performed by ANOVA followed by the Bonferroni–Dunn test for multiple comparisons, and statistical significance indicated as *P < 0.05, **P < 0.001.
Treatments reduce cytokine accumulation in the CNS

Local expression of a large panel of cytokines in the CNS at day 75 was analysed by a non-radioactive RPA using RNA extracted from spinal cord homogenates (Fig. 8A) and by ELISA (Fig. 8B) of supernatants of spinal cord lysates. Only IL-4 (interleukin-4) and TNF-α (tumour necrosis factor-α) mRNAs were found expressed at readily detectable, but low levels in the CNS of normal undiseased mice (Fig. 8A). Levels of all cytokine mRNAs were greatly elevated in the CNS of diseased mice, in particular those encoding IFN-γ (interferon-γ), IL-8, IL-12 and TNF-α, but also the anti-inflammatory cytokine TGF-β (transforming growth factor-β). The various treatments had variable effects on the presence of each cytokine RNA. Thus, GPE and NBQX had little or no effect on IL-5 mRNAs, whereas anti-MAdCAM-1 antibody reduced levels by 50%. Anti-MAdCAM-1 mAbs and the two combinational treatments reduced IL-10 mRNA levels to 25–50%. GPE and NBQX in combination were very effective at inhibiting (by 85%, \( P < 0.001 \)) levels of TGF-β mRNAs. Each treatment reduced IL-8 and 12 mRNA levels by ~60% and 30%, respectively, but the combinations did not exhibit synergy. In almost every instance, anti-MAdCAM-1 mAb was more effective than GPE and NBQX. The triple combination was more effective than the other treatments in inhibiting IL-6, TNF-α, IL-10 and IL-4.

IL-4, IL-6, IL-10, IL-12, TNF-α and IFN-γ proteins were detected at low levels in normal healthy mice by ELISA and increased markedly in diseased mice by day 75 (Fig. 8B). Only IL-5 was completely undetectable in healthy mice. ELISA confirmed that each treatment was able to reduce the levels of CNS cytokines in EAE mice at day 75 by 10–95%. Thus, the levels of IL-12 (65–90%), IL-5 (62–75%), IL-6 (70–95%) and IL-10 (55–75%) were strongly down-regulated, whereas reductions in IL-4 (10–45%), TNF-α (15–60%) and IFN-γ (30–60%) were less spectacular except in the case of the triple therapy. The results obtained by RPA and ELISA were generally comparable in that each treatment reduced cytokine levels, but the degree of down-regulation of cytokine mRNAs versus protein did not necessarily correlate (for example IL-5, IL-12 and IFN-γ), which could reflect the labilities of the respective RNA versus protein molecules. Expression of nitric oxide was dramatically increased in EAE mice, and significantly reduced in response to all treatments concomitant with significantly reduced levels of the latter proinflammatory cytokines.

Combination therapy suppresses inflammation

The effect of each treatment form on CNS inflammation was determined by scoring leukocyte infiltration into spinal cords at different time points before (day 45) and following (days 60, 65, 70 and 119) treatment (Table 3). As reported previously (Kanwar et al., 2000a), anti-MAdCAM-1 mAb treatment rapidly reduced leukocyte infiltration into the CNS, such that at day 70 there were few inflammatory cells proximal to the meninges. Surprisingly, the combination of NBQX and GPE rapidly reduced inflammation to a level similar to that achieved with the anti-MAdCAM-1 mAb, whereas GPE and NBQX monotherapies had little effect until day 119. As expected, the combination of all three reagents was the most effective at reducing CNS inflammation, giving the very low total inflammation score of 1 from day 65 of treatment, compared with 6 to 7 for PBS-treated and rat IgG-treated controls.

Anti-MOG antibody response

Sera from all EAE mice produced anti-MOG35-55 antibodies against the immunizing MOG35-55 peptide, which is an established antibody target in C57BL/6 mice (Huang et al., 2001) (Fig. 9). GPE and NBQX alone or in combination had little affect (\( P > 0.05 \)) on anti-MOG antibody levels despite their ability to inhibit disease severity, but in accord with the fact that Ig is not thought to play an important pathogenic role in MOG35-55 peptide-induced EAE in C57BL/6 mice (Hjelmstrom et al., 1998). Nevertheless, anti-MAdCAM-1 mAb either alone (\( P < 0.001 \)) or in combination (\( P < 0.001 \)) reduced anti-MOG35-55 antibody levels by 60–70%.

### Table 3 Inflammation status scale

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Inflammation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 45</td>
</tr>
<tr>
<td>PBS</td>
<td>3</td>
</tr>
<tr>
<td>Rat IgG</td>
<td>3</td>
</tr>
<tr>
<td>GPE</td>
<td>2</td>
</tr>
<tr>
<td>NBQX</td>
<td>3</td>
</tr>
<tr>
<td>GPE + NBQX</td>
<td>2</td>
</tr>
<tr>
<td>MAdCAM-1</td>
<td>3</td>
</tr>
<tr>
<td>GPE + NBQX + MAdCAM-1</td>
<td>3</td>
</tr>
</tbody>
</table>

*Scoring and averaging in two selected animals was determined from two coded sections, read blindly, from each cord level at days 45, 60, 65, 70 and 119. TTotal score from day 65.*
effective, it is necessary to understand the mechanisms responsible for disease progression. The inflammatory insult leads to secretion of large amounts of glutamate from activated leukocytes, neurons and microglia (Piani et al., 1991). Activated immune cells produce glutamate in large quantities by deamidating glutamine via glutaminase (Werner et al., 2000). Glutamate excitotoxicity mediated by AMPA/kainate types of glutamate receptors damages not only neurons, but also myelin producing oligodendrocytes. Oligodendrocytes have only the AMPA/kainate glutamate receptor and are exquisitely vulnerable to glutamate excitotoxicity, whereas neurons have both types of receptor (Yoshioka et al., 1996; Gill et al., 2000). Thus, NBQX might be expected to protect both oligodendrocytes and neurons from glutamate-mediated excitotoxicity by blocking AMPA/kainate receptors, but alone would not be sufficient to completely protect neurons. GPE might provide the added protection for neurons. In accordance with this theory, Pitt et al. (2000) demonstrated that NBQX spares axons and protects oligodendrocytes during the course of EAE; GPE also prevents neuronal cell death in the hippocampus injured by NMDA. GPE has been previously shown to interact with NMDA-type glutamate receptors (Bourguignon et al., 1994; Ikeda et al., 1995; Saura et al., 1999), but most studies report this interaction occurs at μM concentrations. After an hypoxic–ischaemic acute brain injury in the rat, administration of neuroprotective doses of GPE achieve nM levels of GPE in cerebrospinal fluid (P. D. Gluckman, personal communication), suggesting that NMDA receptor antagonism is not involved in this model. In EAE, the blood–brain barrier is disrupted allowing the entry of macromolecules, suggesting administered GPE may reach the CNS in higher amounts. NBQX and GPE were similarly effective at reducing the clinical score, and both decreased the apoptosis of neurons, particularly in combination. It was surprising that GPE was as effective as NBQX at preventing the loss of oligodendrocytes, though both agents were weakly effective unless used in combination. GPE has the ability to up-regulate the expression of GAD (Sara et al., 1989), which could help diminish the toxic levels of glutamate accumulating in the CNS and thereby indirectly prevent loss of oligodendrocytes. The triple treatment was superior to all other treatments in reducing the apoptosis of neurons and oligodendrocytes in the CNS.

Increased concentrations of glutamate have been shown to induce a dramatic increase in the Na+-dependent glutamate transporter EAAC1 and concomitant decreases in the transporters GLAST and GLT-1 during EAE in rats. Changes in glutamate transporters, which may play a critical role in pathological changes and neuronal dysfunction in EAE, are suppressed by treatment with NBQX (Ohgoh et al., 2002). Our results indicate that NBQX is also particularly effective at suppressing the levels of GluR2 during EAE, which would otherwise be expected to respond to increased levels of glutamate.

**Discussion**

Our results demonstrate that short-term antibody blockade of MAdCAM-1 is able to completely prevent the early development of EAE. Anti-MAdCAM-1 mAb treatment blocked T cell infiltration into the CNS, and hence prevented the influx of new inflammatory cells that are required to replace those undergoing apoptosis, as proposed for anti-α4 integrin mAb-mediated recovery (Hyduk and Karlik, 1998). Thus, anti-MAdCAM-1 mAb blockade led to a significant reduction in the overall apoptotic index. In support, lymphocytes in gene knockout mice deficient in integrin α4β7, the major receptor for MAdCAM-1, fail to arrest and adhere to the vasculature (Wagner et al., 1996). In contrast, short-term GPE and NBQX monotherapies could only suppress the development of EAE, such that disease gradually relapsed over several months. Nevertheless, it was surprising that the combination of GPE and NBQX synergized so effectively to suppress disease for almost 2 months. In the treatment of advanced stage EAE, short-term and prolonged repetitive anti-MAdCAM-1 antibody blockade was ineffective and, while NBQX and GPE monotherapies diminished disease severity, they could not prevent partial hind limb paralysis. In contrast, the disease ameliorated in mice treated with a combination of GPE and NBQX, and/or NBQX + GPE + anti-MAdCAM-1 mAb, such that the only noticeable disease symptom retained was slightly flaccid tails. Notably, the disease relapsed when treatment was suspended, suggesting treatment must be maintained to be effective. The latter combinational treatments led to reduced neuropathology, inflammation, axonal damage and loss of oligodendrocytes, and conversely increased remyelination, and mice regained body weight.
It was surprising that GPE and NBQX synergized to reduce inflammation to levels almost equivalent to that achieved with anti-MAdCAM-1 mAb therapy. A direct effect of NBQX and NMDA antagonist on the inflammatory response has already been ruled out by previous studies (Wallstrom et al., 1996; Pitt et al., 2000; Smith et al., 2000; Werner et al., 2000) and hence this possibility was not examined here. Thus, NBQX does not alter the proliferative activity of antigen primed T cells (Pitt et al., 2000; Smith et al., 2000; Werner et al., 2000). Similarly, NMDA antagonists do not affect lymphocyte proliferation or cytokine secretion in response to encephalitogenic autoantigen (Wallstrom et al., 1996). However, it has recently been demonstrated that T cells express high levels of the glutamate ion channel receptor GluR3. Glutamate triggered T cell adhesion to laminin and fibronectin, and up-regulated the chemotactic migration of T cells toward the chemokine SDF-1α (stromal cell derived factor-1α), which is constitutively expressed in the nervous system (Ganor et al., 2003). It has been proposed that glutamate and SDF-1α act in concert to recruit T cells to the CNS, in accord with the fact that laminin plays a crucial role in recruiting autoaggressive T cells. It has further been proposed that NBQX suppresses EAE because not only does it block glutamate/AMPA receptors expressed on neurons and glia, but it also blocks AMPA receptors expressed on encephalitogenic T cells—thereby reducing T cell activation. The ability of NBQX and GPE to reduce inflammation may also reflect their abilities to prevent the apoptosis of neurons, which would otherwise lead to a heightened inflammatory response due to release of autoantigen and a requirement to clear the damage done. In this scenario, damage to neurons and oligodendrocytes would be seen to be a major perpetuator of the inflammatory response. Lombardi et al. (2001) have revealed that AMPA/kainate and NMDA antagonists inhibit glutamate-stimulated lymphocyte proliferation; thus, NBQX and GPE may indirectly inhibit inflammation. Inhibition of inflammation would lead to lowered levels of glutamate, which would in itself be beneficial as low doses of glutamate are neuroprotective against high toxic doses of glutamate (Jonas et al., 2001).

Certain proinflammatory cytokines have been shown to be critical to the progression of EAE. At first sight, our results seem potentially discrepant in that we observed dramatic increases in all proinflammatory cytokines examined in the spinal cords of EAE mice including IL-1, IFN-γ, IL-6, IL-8, IL-12 and TNF-α but, in addition, immunoregulatory cytokines that might be expected to attenuate EAE including IL-10, IL-4 and TGF-β (Samoilova et al., 1998). However, the concomitant induction of both proinflammatory and immunoregulatory cytokines has been reported to be a feature of MOG-induced EAE (Okuda et al., 1998a), whereas in contrast IL-10, IL-4 and TGF-β are not increased in MBP-induced relapsing EAE (Okuda et al., 1998b). Mice that are resistant to MOG-induced EAE secrete primarily IL-4/IL-10 and TGF-β, and have decreased levels of IFN-γ (Maron et al., 1999). It has been proposed that the disease course of EAE may be influenced by the interplay between the proinflammatory and immunoregulatory cytokines (Okuda et al., 1998a). IL-6 in particular appears to play an obligatory role in EAE, as anti-IL-6 antibodies reduce the incidence and severity of EAE in SJL mice immunized with mouse spinal cord homogenate (Gijbels et al., 1995) and IL-6-deficient gene knockout mice are completely resistant to MOG35-55 induced EAE (Eugster et al., 1998). In accord with previous studies (Diab et al., 1997), we found that IL-6 mRNA and protein were highly expressed in the spinal cords of mice with EAE, whereas the spinal cords of healthy controls contained only trace levels of IL-6 protein. Each of the treatments strongly decreased the expression of IL-6 in the spinal cords of EAE mice, which alone might be expected to have a profound effect on disease remission. All cytokine proteins were down-regulated in response to each treatment, though IFN-γ and IL-4 were affected to a lesser extent. IL-10 was similarly down-regulated, suggesting remission was primarily due to the down-regulation of proinflammatory cytokines rather than an altered balance of pro-and anti-inflammatory cytokines. Interestingly although IL-12 expression was increased in diseased mice and down-regulated during remission, IL-12 responsiveness is not required in the pathogenesis of inflammatory demyelination in the CNS (Murphy et al., 2003; Zhang et al., 2003). Our results are in accord with those of Okuda et al. (1998b), which showed that mRNA levels for both inflammatory cytokines including interleukin IL-1α, IL-2, IL-6, IFN-γ, TNF-α and TNF-β and immunoregulatory cytokines including IL-4, IL-10 and TGF-β were up-regulated in EAE and down-regulated in the recovery phase of EAE. They concluded that the relative reduction in production of TGF-β or IL-6 in the peripheral circulation might participate in the induction or remission of EAE, respectively.

Nitric oxide biosynthesis is elevated in the CNS in multiple sclerosis and EAE in response to activation of astrocytes, macrophages and microglia by the proinflammatory cytokines IFN-γ, TNF-α and IL-1β (Okuda et al., 1995; Parkinson et al., 1997). Given that nitric oxide is implicated in the pathology of multiple sclerosis and EAE (MacMicking et al., 1992; Owens et al., 2002), it is potentially significant that expression of this detrimental metabolite was dramatically increased in EAE mice, and significantly reduced in response to all treatments concomitant with significantly reduced levels of RNA and/or protein for the latter proinflammatory cytokines.

Autoantibodies against MOG can be found within acute lesions of human multiple sclerosis and animal EAE, where they may contribute to the disintegration of myelin sheaths (Genain et al., 1999). Significant positive correlations have been found between anti-MOG35-55 antibody levels in EAE and clinical scores (Costa et al., 2003). None of the treatments with GPE and NBQX significantly inhibited the levels of anti-MOG35-55 antibodies, whereas those including anti-MAdCAM-1 mAb significantly blocked anti-MOG35-55 antibody production. The relevance of these observations to
disease remission is not clear as B cell deficient mice are still fully susceptible to MOG_{35-55} induced EAE (Eugster et al., 1999). Several subtypes of GluR are widely distributed outside the CNS in peripheral tissues, but their roles have not been defined (Gill et al., 2000). Thus, the presence of GluR subtypes was demonstrated in the rat and monkey heart, with preferential distribution within the conducting system, nerve terminals and cardiac ganglia. NMDAR 1, GluR 2/3, and mGluR 2/3 are also present in kidney, liver, lung, spleen and testis (Gill et al., 2000). The latter might be anticipated to complicate therapy. However, disability scores correlated well with the clinical scores of paralysis, such that animals treated with the combination of NBQX and GPE in the presence or absence of anti-MAdCAM-1 mAb displayed discernible improvements in all physical features examined including alertness, spontaneous mobility, tone, motor function (grip), sensory function, weight and shiny hair/skin firmness. The triple combination afforded the greatest improvement in the disability scores. No discernible changes to any of the major organs were detected following autopsy (data not shown).

In conclusion, we have shown for the first time that a multi-faceted approach, which inhibits the inflammatory cascade and/or simultaneously protects neurons and oligodendrocytes from damage, can effectively attenuate advanced stage demyelinating disease in a chronic progressive model of multiple sclerosis. It is known that there is relative sparing of axons in multiple sclerosis, and that the CNS can repair itself (Lassmann et al., 2001). The results of the multi-faceted approach have implications for the treatment of multiple sclerosis, as they suggest it might be possible to significantly limit ongoing damage so that axons can be repaired.

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