Long-term protection of central axons with phenytoin in monophasic and chronic-relapsing EAE

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Axonal degeneration is a major contributor to non-remitting deficits in multiple sclerosis, and there is thus considerable current interest in the development of strategies that might prevent axonal loss in neuroinflammatory disease. Dysregulation of sodium ion homeostasis has been implicated in mechanisms leading to axonal degeneration, and several studies have shown that blockade of sodium channels can ameliorate axon damage following anoxic, traumatic and nitric oxide-induced CNS injury. Two sodium channel blockers, phenytoin and flecainide, have been reported to protect axons in experimental autoimmune encephalomyelitis (EAE) for 30 days, but long-term protective effects have not been studied. We demonstrate here that oral administration of phenytoin provides long-term (up to 180 days) protection for spinal cord corticospinal tract (CST) and dorsal column (DC) axons in both monophasic (C57/BL6 mice) and chronic-relapsing (Biozzi mice) murine EAE. Untreated C57/BL6 mice exhibit a 40–50% loss of CST and DC axons at 90 and 180 days post-EAE induction via myelin-oligodendrocyte glycoprotein (MOG) injection. In contrast, only 4% of DC axons are lost at 90 days, and only 8% are lost at 180 days in phenytoin-treated C57/BL6 mice with EAE; only 21–29% of CST axons are lost at 90 and 180 days in phenytoin-treated C57/BL6 mice with EAE. Attenuation of dorsal column compound action potentials was ameliorated and clinical status was also significantly enhanced with phenytoin treatment at 90 and 180 days in this model. In addition, inflammatory cell infiltration into the dorsal columns was reduced in phenytoin-treated mice with EAE compared with untreated mice with EAE. Similar results were obtained in Biozzi mice with chronic-relapsing EAE followed for 120 days post-injection. These observations demonstrate that phenytoin provides long-term protection of CNS axons and improves clinical status in both monophasic and chronic-relapsing models of neuroinflammation.

Keywords: axon protection; EAE; inflammatory cell; multiple sclerosis; phenytoin; sodium channel

Abbreviations: EAE = experimental autoimmune encephalomyelitis; CAP = compound action potential; CST = corticospinal tract; DC = dorsal columns; DF = dorsal funiculus


Introduction

It has become increasingly clear that degeneration of CNS axons occurs frequently in multiple sclerosis (Ferguson et al., 1997; Trapp et al., 1998), that it can occur early in the clinical course of the disease (Filippi et al., 2001), and that it is a major contributor to non-remitting, persistent deficits (Davie et al., 1995; Bjartmar et al., 2000; Lee et al., 2000). Thus, there has been increasing interest in protective strategies that might preserve axonal integrity and maintain axonal conduction in multiple sclerosis. Early studies suggested that molecular mechanisms of axonal injury in multiple sclerosis differ from mechanisms that injure neuronal cell bodies, dendrites and other synaptic structures (Waxman et al., 1991). These studies demonstrated that a persistent sodium influx can drive reverse Na⁺/Ca²⁺ exchange into white matter axons after anoxic insult, importing high levels of calcium that lead to irreversible
axonal injury (Stys et al., 1992), and subsequently showed that non-inactivating sodium channels, capable of carrying this persistent sodium current, are present within white matter axons (Stys et al., 1993). Fern et al. (1993) showed that clinically used sodium channel blockers, including phenytoin, can protect CNS white matter axons in vitro after insults such as anoxia. Kapoor et al. (2003) demonstrated that nitric oxide (NO), which is present at increased levels within multiple sclerosis lesions (Bo et al., 1994; Brosnan et al., 1994; Smith et al., 1999; Smith and Lassmann, 2002), can produce axonal disruption, possibly via mitochondrial injury that triggers a similar molecular cascade involving sodium channels, and demonstrated that these axons can be protected from NO-mediated damage with sodium channel blockers.

Extending these in vitro observations to an in vivo model of multiple sclerosis, two studies have examined the effects of sodium channel blockers in experimental autoimmune encephalomyelitis (EAE). Lo et al. (2003) studied the effects of phenytoin, administered orally so as to achieve plasma levels within the therapeutic range used in humans with epilepsy, in an EAE model with a monophasic course in C57/BL6 mice, and demonstrated amelioration of axonal loss, maintenance of axonal conduction, and improved behavioural status for 30 days. Bechtold et al. (2004) studied the effects of another sodium channel blocker, flecainide, in chronic-relapsing EAE in the dark agouti (DA) rat, and observed similar protection of axons for 30 days. These studies strongly suggest a major contribution of sodium channels in the cascade of events leading to axonal degenerations in inflammatory/demyelinating disorders (for review, see Bechtold and Smith 2005).

Thus far, studies of sodium channel blockers have examined their effects for only 30 days in EAE. However, there is some evidence suggesting that mechanisms of axonal injury may differ during early and later stages of disease (see, e.g. Bjartmar et al., 2003), implying that longer-term studies are needed. Moreover, recent evidence indicates that, in addition to a direct protective effect on axons, sodium channels play a role in activation of microglia and macrophages in multiple sclerosis, and shows that phenytoin can substantially reduce the inflammatory response in EAE (Craner et al., 2005), raising the possibility that sodium channel blockade may have an acute effect in EAE, attenuating the active inflammatory phase of disease. For these reasons, and because several clinical studies on sodium channel blockers are planned in patients with multiple sclerosis, we have carried out long-term studies in which we have assessed the number of CNS axons, conduction along them, and clinical progression, as well as the degree of inflammatory cell infiltration, for as long as 180 days in two models of EAE, one monophasic and the other chronic-relapsing. Our results demonstrate that long-term administration of phenytoin produces sustained protective effects on axons within the spinal cord, and reduces the degree of inflammatory cell infiltration, in mice with both forms of EAE.

**Material and methods**

**Induction of EAE**

Experiments were carried out in accordance with NIH guidelines for the care and use of laboratory animals; all animal protocols were approved by the IACUC of VA Connecticut Healthcare System, West Haven, CT. C57/BL6 (Harlan, Indianapolis, IN) and Biozzi (Harlan Sera-Lab Ltd, Loughborough, UK) mice 6–10 weeks of age were injected subcutaneously in the flank with 200 µl of an emulsion of 300 µg of rat myelin-oligodendrocyte glycoprotein (MOG) 35–55 peptide (W. M. Keck Biotechnology Resource Center, Yale University) in incomplete Freund’s adjuvant (IFA; Sigma, St Louis, MO) supplemented with 500 µg of *Mycobacterium tuberculosis* H37Ra (Difco, Detroit, MI), as described by Lo et al. (2003) and Craner et al. (2004a). The MOG injection, with mycobacterium supplemented IFA, was repeated in the other flank 1 week later. The mice also received an injection of 500 ng pertussis toxin (Sigma) in 200 µl PBS intraperitoneally (i.p.) immediately after the first immunization with MOG and then again 48 h later. In agreement with previous descriptions, the C57/BL6 mice developed a monophasic clinical course (Lassmann 1993), while the Biozzi mice exhibited a chronic-relapsing clinical phenotype (Baker et al., 1990).

Immunized mice were observed daily for clinical signs and scored as described by Lo et al. (2003) on a 0–6 scale, with 0.5 gradations for intermediate scores, as follows: 0 = normal without clinical signs; 1 = flaccid tail; 2 = abnormal righting reflex; 3 = partial hindlimb paralysis; 4 = complete hindlimb paralysis; 5 = moribund; and 6 = death. A total of 38 C57/BL6 and 18 Biozzi mice were injected with MOG and observed. Of these mice, six C57/BL6 and two Biozzi mice died prior to initiation of phenytoin treatment (see below). Of the remaining 32 C57/BL6 mice, 17 with EAE were placed in the untreated group and 5 of these died prior to the termination of the study; 15 C57/BL6 mice with EAE were administered phenytoin and 5 died prior to the completion of the study. Of the 16 Biozzi mice, 9 with EAE were placed in the untreated group and 3 died prior to termination of the study; 7 Biozzi mice with EAE were administered phenytoin and 1 died prior to completion of the study.

**Phenytoin treatment**

Beginning on day 10 following the initial MOG injection, pelleted mouse chow incorporating phenytoin (5.5 diphenylhydantoin; Sigma) was fed to EAE-induced mice; a separate group of EAE mice were fed the identical standard mouse chow not containing phenytoin. Serum phenytoin levels were measured from each group of mice on the day of perfusion and electrophysiological study (C57/BL6: Days 90 and 180; Biozzi: Day 120). Mean serum phenytoin levels were: C57/BL6 at Day 90 = 19.9 ± 0.4 and at Day 180 = 19.8 ± 1.7 µg/ml; Biozzi at Day 120 = 11.3 ± 1.4 µg/ml. These serum phenytoin levels are within the therapeutic range in the clinical setting (10–20 µg/ml).

**Immunocytochemistry**

Normal, EAE and EAE+phenytoin mice were perfused through the heart with a phosphate-buffered saline (PBS) and then with 4% paraformaldehyde in 0.14 M Sorensen’s phosphate buffer. Spinal cords were carefully excised, cryoprotected with 30% sucrose in PBS and frozen and 8 μm cross-sections of the mid-cervical spinal cords (ensuring that similar regions were analysed for all...
conditions) were cut. The cervical spinal cord was chosen for this analysis to permit comparison with an earlier study on EAE (Lo et al., 2003). Sections were incubated with antibodies against phosphorylated neurofilament (SMI-31; 1: 20 000; Sternberger Monoclonals Inc., Lutherville, MD) and non-phosphorylated neurofilament (SMI-32; 1: 20 000) overnight at 4°C on a rotating shaker. Sections were then incubated sequentially in: (i) PBS, (ii) goat anti-mouse IgG-biotin (1: 1000, Sigma, St Louis, MO), (iii) PBS, (iv) avidin-HRP (1: 1000; Sigma), (v) PBS and (vi) heavy metal enhanced diaminobenzidine (DAB; Pierce, Rockford, IL). In order to label inflammatory cells within the dorsal funiculi of normal, EAE and EAE+phenytoin mice, cervical spinal cord sections were incubated with rat anti-mouse CD-45 (1: 10; Caltag, Burlingame, CA), a marker for leucocyte common antigen. Sections were incubated with antibodies against neurofilament (SMI-32; 1: 20 000) overnight at 4°C to have no effect on electrophysiological properties of normal spinal cord (Hains et al., 2004). For quantification of inflammatory cells within the dorsal funiculus (DF) in normal, EAE and EAE+phenytoin mice, one spinal cord section was randomly selected from 6–8 sections on the slide for each mouse and for each condition, and every CD45+ cell within the extent of the DF was manually counted. The boundaries of the DF were circumscribed, and the area was calculated utilizing MetaVue software (Molecular Devices, Downingtown, PA), as previously described (Lo et al., 2003).

For quantification of axons in normal, EAE and EAE+phenytoin spinal cords, sections were examined with a Nikon E800 microscope equipped with a 100x oil objective, and digital images were captured with a CoolSNAP HQ camera (Photometrics, Tucson, AZ). Axons were visually identified within preselected fields (500 μm² in area) at specific sites within the dorsal funiculus (cuneate fasciculus) and dorsal corticospinal tract (CST) and counted utilizing MetaVue software (Molecular Devices, Downingtown, PA), as previously described (Lo et al., 2003). For quantification of inflammatory cells within the dorsal funiculus (DF) in normal, EAE and EAE+phenytoin mice, one spinal cord section was randomly selected from 6–8 sections on the slide for each mouse and for each condition, and every CD45+ cell within the extent of the DF was manually counted. The boundaries of the DF were circumscribed, and the area was calculated utilizing MetaVue software; data are presented as number of CD45+ cells per dorsal funiculus and also number of CD45+ cells per 10⁶ μm².

The number of mice analysed for each condition was as follows: 90 day C57/BL6: normal = 4; EAE = 5; EAE+phenytoin = 5; 180 day C57/BL6: normal = 5; EAE = 5; EAE+phenytoin = 5; 120 day Biozzi: normal = 3; EAE = 5; EAE+phenytoin = 6. Statistical comparisons between normal and experimental groups were performed with two-sample t-tests utilizing Microsoft Excel software, with the criterion for significance at \( P < 0.05 \).

Electrophysiological procedures
Normal, EAE and EAE+phenytoin C57/BL6 and Biozzi mice underwent compound action potential (CAP) recordings from dorsal columns according to established methods (Lo et al., 2003; Hains et al., 2004). The experimenter was blinded to the status of the animals. Mice were initially anaesthetized with halothane (3% in a Plexiglas box), and deep anaesthesia was maintained with 1.2% halothane through an anaesthesia mask and verified by the absence of a withdrawal reflex to noxious peripheral pinch. Heart rate (~360 beats/min) was carefully monitored, and body temperature was regulated by a feedback-controlled thermal blanket set to 37°C. Mice were fixed in a stereotaxic apparatus (Kopf Instruments, Tujunga, CA). A midline incision was made through the skin, a laminectomy was performed, and the dura was carefully cut to expose the lower thoracic and lumbar spinal cord regions. The spinal cord was bathed in warm (37°C) mineral oil.

Silver wire electrodes (0.01 inch diameter; A–M Systems, Carlsborg, WA) insulated except at the tip were used for stimulation of dorsal column axons at L1–L2 and recording of CAP at T5–T6 at the surface of the midline spinal cord. PANCuronium bromide (0.3 mg/kg, Sigma) was injected i.p. to minimize muscular contractions at the time of CAP recordings. The signal from the recording electrode was amplified with filters set at 300–3000 Hz and output set to positive polarity (DAM 80, WPI, Sarasota, FL), collected (CED 1401+, Cambridge Electronic Design, UK), and a computer was used for data analysis (Spike2 software v5.03, Cambridge Electronic Design). Single current pulses (0.05 ms, with 3 s inter-pulse period) were applied through an isolated pulse stimulator (2100, A–M Systems) in increasing increments (0.1–1.1 mA). The CAP peaks of interest in the sampled recordings corresponded well to the expected latency of CAP response. The peak-to-peak amplitude of CAP was calculated as the value between the positive (first) and negative (second) peaks of the biphasic wave, as previously described by Lo et al. (2003).

The area of the CAP was calculated by rectifying the negative component (full-wave rectification using Spike 2 script software, Cambridge Electronic Design) and measuring its area. For each animal, four sets of recordings (runs) were collected by sequential stimulation at intensities ranging from 0.1 to 1.1 mA in 0.1 mA increments. Although most sets of recordings displayed increasingly large CAPs that reached a clear plateau, occasional runs showed noise in CAP amplitudes due to respiration or electrode movement, and did not exhibit a plateau. Sets of recordings with plateaus, indicating supramaximal responses at higher stimulation intensities (typically >0.8 mA), were used for analysis, and the values from these runs (typically \( n = 2–3 \) runs/animal) at each stimulus intensity were averaged. At the end of each experiment, the dorsal half of the spinal cord was transected between stimulating and recording electrodes to confirm that a CAP could not be detected. As assessed by CAP recordings, phenytoin treatment appeared to have no effect on electrophysiological properties of normal spinal cord (Hains et al., 2004).

For CAP data analyses, the number of mice recorded for each condition was: 90 day C57/BL6: normal = 3; EAE = 5; EAE+phenytoin = 5; 180 day C57/BL6: normal = 3; EAE = 7 (from which axon counts were obtained from five randomly-selected mice); EAE + phenytoin = 5; 120 day Biozzi: normal = 3; EAE, 6 (from which axon counts were obtained from five randomly-selected mice); EAE + phenytoin = 6.

Results
To examine whether administration of the sodium channel blocker phenytoin provides long-term (up to 180 days) protection of CNS axons in monophasic murine EAE, as we have demonstrated in the short-term (30 days; Lo et al., 2003), we determined the density of mid-cervical spinal cord axons, the amplitude and area of the CAP of spinal cord axons, and the neurological status of C57/BL6 mice with and without oral administration of phenytoin at 90 and 180 days post-induction of EAE. In addition, to determine whether phenytoin provides protection of CNS axons in chronic-relapsing murine EAE, we performed similar studies with Biozzi mice.
### Monophasic EAE

#### Axonal counts

The number of mid-cervical spinal cord axons was counted within standardized 500 μm² fields at predetermined sites in the DF and dorsal CST in normal, EAE and EAE+phenytoin C57/BL6 mice at 90 and 180 days post-MOG injection. To identify all axons within these regions, sections were reacted with antibodies generated against both phosphorylated (SMI-31) and non-phosphorylated (SMI-32) neurofilaments (Lovas et al., 2000; Lo et al., 2002, 2003). Representative images of the axons from the different experimental groups are shown in Fig. 1; a substantial reduction in the density of axons in both DF and corticospinal tracts in EAE mice at 90 and 180 days is evident. The administration of phenytoin to EAE mice ameliorated the loss of axons in DF and CSTs at both 90 and 180 days, such that the number of axons in the EAE+phenytoin mice approaches that of normal mice (Fig. 1).

Axonal counts for the normal, EAE and EAE+phenytoin mice at 90 and 180 days post-MOG injection are presented in Table 1. These results demonstrate that EAE mice exhibited a 40–50% loss of DF and CST axons compared with normal mice at 90 and 180 days post-MOG injection, and that phenytoin administration had a significant protective effect on these axons at both 90 and 180 days. At 90 days, the number of DF axons per 500 μm² field in EAE+phenytoin mice was 96% of the number in normal EAE mice, while at 180 days the number of DF axons in EAE+phenytoin mice was 92% of the number in normal mice. In the CST, phenytoin administration produced substantial neuroprotective effects, although not as robust as within DF, with the density of corticospinal axons in EAE+phenytoin mice being 73% of the number in normal mice at 90 days, and 79% at 180 days.

#### Spinal cord electrophysiology

Representative CAP from spinal cords of normal, EAE, and EAE+phenytoin C57/BL6 mice at 180 days are shown.
in Fig. 2B; the CAP for the untreated EAE mouse is substantially smaller than that of the normal mouse.

At 90 days post-MOG-injection, untreated C57/BL6 EAE mice had significantly reduced CAP amplitudes (0.15 ± 0.07–0.47 ± 0.03 mV) throughout the stimulation range (0.2–1.1 mA) compared with normal mice which exhibited CAP amplitudes of 0.34 ± 0.02–1.03 ± 0.06 mV (Fig. 2C). In comparison to untreated EAE mice, EAE+phenytoin mice displayed significantly (P < 0.05) larger CAP amplitudes (0.28 ± 0.03–0.78 ± 0.04 mV) throughout the same stimulation range (0.2–1.1 mA) (Fig. 2C). We observed a supramaximal CAP response which was evident by 0.8–0.9 mA in most sets of CAP recordings. At this intensity, the CAP amplitudes of untreated EAE and EAE+phenytoin mice were 45 and 75% of CAP amplitudes in normal mice, respectively. Similar to
CAP amplitudes, CAP areas were significantly reduced at stimulation intensities of 0.5–1.1 mA in untreated EAE mice (0.14 ± 0.60–0.19 ± 0.05 mV ms) compared with normal mice (0.30 ± 0.03–0.38 ± 0.04 mV ms) (Fig. 2D). The CAP areas of EAE+phenytoin mice (0.21 ± 0.03–0.27 ± 0.03 mV ms) were significantly ($P < 0.05$) larger than CAP areas of untreated EAE mice. At 1.1 mA stimulus intensity, the CAP areas of untreated EAE and EAE+phenytoin mice were 50 and 72% of the normal CAP area, respectively.

At 180 days post-MOG injection, there were similar differences in CAP amplitudes and areas between normal, untreated EAE and EAE+phenytoin mice. Normal mice displayed CAP amplitudes of 0.19 ± 0.04–1.04 ± 0.09 mV at 0.1–1.1 mA stimulation intensities, while EAE mice exhibited significantly lower CAP amplitudes (0.09 ± 0.02–0.50 ± 0.08 mV) at all stimulation intensities (Fig. 2E). The CAP amplitudes of EAE+phenytoin mice (0.18 ± 0.03–0.77 ± 0.06 mV) were significantly ($P < 0.05$) increased compared with untreated EAE mice within the entire stimulation intensity range (Fig. 2E). At 1.1 mA stimulus intensity, CAP amplitudes of untreated EAE and EAE+phenytoin mice were 48 and 74%, respectively, of CAP amplitudes in normal mice. We observed supramaximal responses in all animals, typically at stimulation intensities >0.8 mA.

Differences in CAP areas between normal, EAE and EAE+phenytoin mice paralleled the differences in CAP amplitudes for these groups of mice (Fig. 2F). CAP areas were significantly ($P < 0.05$) decreased in untreated EAE mice (0.17 ± 0.02–0.18 ± 0.04 mV ms) compared with normal mice (0.37 ± 0.03–0.39 ± 0.04 mV ms) within the 0.7–1.1 mV stimulation intensity range. In comparison to untreated mice, CAP areas for EAE+phenytoin mice (0.28 ± 0.04–0.32 ± 0.05 mV ms) were significantly ($P < 0.05$) increased within the 0.7–1.1 mA stimulation intensity range. At the 1.1 mA stimulus intensity, the CAP areas of EAE and EAE+phenytoin mice were 47 and 81% of normal CAP area, respectively.

**Neurological status**

We assessed the clinical score of each mouse in each experimental group using a rating scale (see Material and methods) that is sensitive to motor function, particularly in the hindlimbs. Normal mice did not exhibit any pathological clinical signs (clinical score = 0). Untreated mice in both the 90 and 180 day experiments displayed a progressive increase in clinical score (worsening clinical status) that plateaued at 25–30 days post-MOG injection at a clinical score of ~3.5 (Fig. 3). In contrast, groups of mice treated with phenytoin, commencing 10 days following MOG injection, exhibited significantly improved clinical scores (1.0–1.5) at 90 days and also at 180 days. These data demonstrate that sustained phenytoin administration provides improved neurological status for substantially longer periods of time than has been previously reported.

**Inflammation**

Because there is evidence suggesting that sodium channels may play a role in activation of microglia/macrophages, and that phenytoin can reduce the inflammatory response in EAE (Craner et al., 2005), we utilized labelling of immune cells with CD45 antibody (Sedgwick et al., 1991), which binds to the leucocyte common antigen, as an index of inflammatory cell infiltration into the dorsal columns of untreated and phenytoin-treated mice with EAE. In normal dorsal columns, few CD45+ cells were observed, while untreated mice at both 90 and 180 days post-MOG injection exhibited substantial numbers of CD45+ immune cells within the dorsal columns (Fig. 4). In contrast, EAE mice treated with phenytoin displayed reduced infiltration of CD45+ immune cells in the dorsal columns at both 90 and 180 days (Fig. 4).
Quantification of the number of CD45⁺ immune cells in untreated and phenytoin-treated EAE mice at 90 and 180 days post-MOG injection is shown in Table 2. The results demonstrate a significant (\( P < 0.05 \)) reduction in the number of CD45⁺ inflammatory cells in the dorsal columns of phenytoin-treated mice compared with untreated mice at 90 days post-MOG injection, while at 180 days there was a substantial, but not statistically significant (\( P < 0.05 \)), reduction in CD45⁺ cell infiltration.

Chronic-relapsing EAE

Axonal counts

EAE in Biozzi mice exhibits a chronic-relapsing course. We asked whether phenytoin provides protection of axons that persists for 120 days in Biozzi mice that were injected with MOG and exhibited at least two relapses. Representative images of spinal cord axons from normal, EAE, and EAE+phenytoin mice are shown in Fig. 5 and demonstrate a substantial reduction in the number of axons in both DF and corticospinal tracts in EAE mice at 120 days. In Biozzi mice that had been treated with...
Phenytoin there was reduced loss of axons in DF and CSTs at 120 days (Fig. 5).

Axonal counts are presented in Table 3. These results demonstrate that Biozzi mice exhibit a 43% loss of DF and 57% loss of CST axons at 120 days post-injection compared with normal mice. Phenytoin administration provided a significant protective effect on these axons; the number of DF axons per 500 μm² in EAE+phenytoin mice was 91% of the number in normal mice, while the number of CST axons in EAE+phenytoin mice was 73% of the number in normal mice.

**Spinal cord electrophysiology**

Normal Biozzi mice exhibited CAP amplitudes of 0.29 ± 0.8–1.23 ± 0.15 mV over the stimulation range, while untreated EAE mice studied at 120 days post-MOG injection displayed significantly reduced CAP amplitudes (0.08 ± 0.07–0.51 ± 0.09) over the same stimulation range (Fig. 6A). In comparison to untreated EAE mice, EAE+phenytoin mice, also studied at 120 days post-MOG injection, exhibited significantly ($P < 0.05$) elevated CAP amplitudes (0.66 ± 0.06–0.86 ± 0.08 mV) within the 0.5–1.1 mA stimulation intensity range. At 1.1 mA stimulus intensity, a significant difference was observed ($P < 0.05$).

**Table 3** Axon count/500 μm² (Biozzi mice at 120 days)

<table>
<thead>
<tr>
<th>Condition</th>
<th>No. of mice (n)</th>
<th>CST (means ± SE)</th>
<th>DF (cuneate fasciculus) (means ± SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>120 days post MOG-injection</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>3</td>
<td>167.3 ± 16.8</td>
<td>57.3 ± 10.4</td>
</tr>
<tr>
<td>EAE</td>
<td>5</td>
<td>73.6 ± 9.2</td>
<td>33.3 ± 2.5</td>
</tr>
<tr>
<td>EAE+phenytoin</td>
<td>6</td>
<td>123.8 ± 8.5*</td>
<td>52.0 ± 5.6*</td>
</tr>
</tbody>
</table>

* $P < 0.05$ compared with untreated EAE.

Fig. 5 Neurofilament staining in dorsal CST and DF of normal, EAE and EAE+phenytoin in Biozzi mice at 120 days post-MOG injection. There is substantial loss of axons in CST and DF in EAE compared with normal mice at 120 days post-injection. In mice treated with phenytoin (EAE+phen), there is a substantial increase in axons within the CST and DF compared with untreated EAE mice.

Fig. 6 CAP amplitude and area in normal, EAE and phenytoin-treated EAE in Biozzi mice. (A) CAP amplitudes for normal, EAE, and EAE+phenytoin in Biozzi mice elicited by stimulus intensities of 0.1–1.1 mA at 120 days post-injection. (B) CAP areas for normal, EAE, and EAE+phenytoin in Biozzi mice elicited by stimulus intensity of 0.1–1.1 mA. Significant differences ($P < 0.05$) between EAE and EAE+P animals are indicated by asterisks.
the CAP amplitudes of untreated EAE and EAE+phenytoin mice were 41 and 69% of normal CAP amplitudes, respectively.

The CAP area was also significantly reduced in untreated EAE mice (0.03 ± 0.05–0.05–0.21 ± 0.03 mV ms) compared with normal mice (0.08 ± 0.03–0.05 ± 0.05 mV ms) throughout 0.3–1.1 mA stimulation intensity range (Fig. 6B). The CAP area of EAE+phenytoin mice (0.26 ± 0.03–0.34 ± 0.04 mV ms) was significantly (P < 0.05) increased, compared with untreated EAE mice (0.17 ± 0.03–0.21 ± 0.03 mV ms) within the 0.6–1.1 mA stimulation range (Fig. 6B). At 1.1 mA, CAP areas for EAE and EAE+phenytoin mice were 46 and 75% of normal CAP areas, respectively.

**Neurological status**

All animals studied displayed at least two relapses. As a result of the relapsing clinical course, there was greater mouse-to-mouse variability within each treatment group than for C57/BL6 mice where the clinical course was much more stable. Nevertheless, at 120 days and at earlier time-points, similar to improvement in clinical status provided by phenytoin in C57/BL6 mice at 90 and 180 days post MOG-injection, phenytoin administration resulted in a significant reduction in the clinical scores (improved neurological status) of MOG-injected Biozzi mice treated with phenytoin compared with untreated EAE mice (Fig. 7). The improvement in clinical scores was evident during both relapses and remissions in these mice and was significant at all times tested, including the longest post-MOG injection interval assessed for the Biozzi model in this study, 120 days.

**Inflammation**

Similar to C57/BL6 mice, the dorsal columns of normal Biozzi mice displayed few CD45+ cells, and untreated Biozzi mice at 120 days post-MOG injection exhibited substantial numbers of CD45+ immune cells within the dorsal columns (Fig. 8). In contrast, Biozzi mice with EAE that were treated with phenytoin displayed reduced infiltration of CD45+ immune cells in the dorsal columns (Fig. 8).

The number of CD45+ immune cells in untreated and phenytoin-treated Biozzi mice with EAE is shown in Table 4. The results demonstrate a significant reduction in the number of CD45+ inflammatory cells in the dorsal columns of phenytoin-treated mice compared with untreated mice.

**Discussion**

The present results demonstrate that the sodium channel blocker phenytoin provides protection for spinal cord axons in long-term monophasic and in chronic-relapsing murine EAE. Phenytoin treatment significantly ameliorated the loss of axons in both forms of EAE throughout the course of this study (up to 180 days for monophasic EAE, 120 days for chronic-relapsing EAE), and the reduced axonal loss was accompanied by a smaller loss of spinal cord
CAP amplitudes and areas in phenytoin-treated mice. Moreover, phenytoin administration significantly improved clinical status of the treated mice for at least 180 days in monophasic EAE and 120 days in chronic-relapsing EAE. In addition, administration of phenytoin resulted in a reduction in the level of inflammatory cell infiltration. Accumulating evidence supports the concept that axonal loss is a major contributor to non-remitting clinical deficits in multiple sclerosis (Bjartmar et al., 2000; Wujek et al., 2002). Axonal damage was detected in both acute and chronic inactive multiple sclerosis plaques, but >10-fold more damaged axons were detected in acute, compared with chronic, lesions (Ferguson et al., 1997; Trapp et al., 1998; Kornek et al., 2002; Kuhlmann et al., 2002), suggesting that axonal loss may be most prominent during the initial inflammatory phase of the disease. In this regard, Wujek et al. (2002) suggested a causal relationship between inflammation, axon loss and neurological disability in relapsing-remitting EAE [SWXJ (H-2q,s) mice]. Consistent with these observations, untreated C57/BL6 mice with monophasic EAE exhibited similar percentage losses of axons in the DF at 30 days post-MOG injection (39%; Lo et al., 2003) and at 90 and 180 days (41 and 40%, respectively; present study); parallel results were observed in the dorsal CST. CAP amplitudes and areas, and clinical scores in mice with monophasic EAE, displayed similar values at 30 days post-injection as at 90 and 180 days. These observations demonstrate that axons are lost early in the course of EAE and emphasize the requirement for protection of axons during the inflammatory/demyelinating phase of the disease.

Prevention of axonal damage in EAE has been reported by several therapeutic agents, including the \( \alpha_1 \)-adrenergic agonist prazosin (White et al., 1992), the immunosuppressant FK506 (tacrolimus; Gold et al., 2004), erythropoietin (Li et al., 2004; Diem et al., 2005), glatiramer acetate (Gilgun-Sherki et al., 2003), calcium channel blockers bepridil and nitrendipine (Brand-Schieber and Werner, 2004) and the sodium channel blockers phenytoin (Lo et al., 2002, 2003) and flecainide (Bechtold et al., 2004). The protective effects of these agents, however, have been demonstrated for only a relatively short term (<45 days). In the earlier studies on sodium channel blockers, phenytoin and flecainide treatment in mice (Lo et al., 2002, 2003) and rats (Bechtold et al., 2004) yielded axonal rescue as assessed at 30 days post-injection. Phenytoin treatment preserved \( \geq 85–90\% \) of optic nerve and spinal cord axons in EAE mice studied at 30 days compared with untreated mice (50–55%; Lo et al., 2002, 2003). Flecainide administration resulted in 83–98% surviving spinal cord axons studied at 28–30 days post-injection compared with 60% in untreated rats (Bechtold et al., 2004). The present study extends these earlier studies and shows for the first time that administration of a sodium channel blocker ameliorates spinal cord axonal loss for a length of time that is a substantial portion of the lifespan of a mouse. Importantly, phenytoin treatment in mice with monophasic and chronic-relapsing EAE

![Figure 8](image-url) Phenytoin reduces the inflammatory reaction in chronic-relapsing EAE. Representative images of dorsal columns at low (top row) and increased (bottom row) magnification from normal, EAE and EAE+phenytoin Biozzi mice at 120 days post-MOG injection. Dorsal columns of normal mice display relatively few CD45\(^+\) immune cells; substantial inflammatory cell infiltration is exhibited in EAE mice. Phenytoin treatment attenuates immune cell infiltrate in EAE. Scale bars, top row, 250 \( \mu \)m; bottom row, 25 \( \mu \)m.

### Table 4 CD45\(^+\) immune cells (Biozzi mice at 120 days)

<table>
<thead>
<tr>
<th>Condition</th>
<th>No. of mice (n)</th>
<th>No. CD45(^+) cells/DF (mean ± SE)</th>
<th>No. CD45(^+) cells/10(^6) ( \mu )m(^2) (mean ± SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>120 days post MOG-injection</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>3</td>
<td>0.3 ± 0.2</td>
<td>0.67 ± 0.4</td>
</tr>
<tr>
<td>EAE</td>
<td>5</td>
<td>24.8 ± 4.5</td>
<td>89.1 ± 13.1</td>
</tr>
<tr>
<td>EAE+phenytoin</td>
<td>5</td>
<td>12.4 ± 1.5*</td>
<td>41.6 ± 4.2*</td>
</tr>
</tbody>
</table>

\*P < 0.05 compared with untreated EAE.
EAE provided partial protection from loss of CAP amplitude in the spinal cord axons and significantly improved clinical status that persisted in these long-term studies. Interestingly, for both C57/BL6 and Biozzi mice, phenytoin treatment provided enhanced protection for axons of the DF compared with the CST (91–97 versus 73–79% of normal number, respectively). While the underlying mechanism responsible for this relative protective difference is unknown, these observations may reflect differences in axonal sizes between the two tracts, with a greater percentage of larger diameter axons in DF, suggesting that phenytoin protection is more effective in larger axons.

How might phenytoin exert its protective effect? Phenytoin is a well-established sodium channel blocker (Ragsdale and Avoli, 1998; Ragsdale et al., 1996) that has been utilized in the treatment of epilepsy and more recently for some pain syndromes. The protective effect of phenytoin on anoxic optic nerve has been ascribed to its sodium channel blocking action on axons (Fern et al., 1993; Stys et al., 1993). The sodium channel blockers tetrodotoxin (Agrawal and Fehlings, 1996; Rosenberg et al., 1999; Teng and Wrathal, 1997; Iwata et al., 2004), procaine (Agrawal and Fehlings, 1996; Rosenberg et al., 1999) and QX314 (Agrawal and Fehlings, 1997) also have protective effects on axons in in vivo and in vitro models of white matter injury, suggesting an involvement of sodium channels in processes leading to axonal injury. Several studies have implicated reverse operation of the axonal Na⁺/Ca²⁺ exchanger (NCX), triggered by an influx of sodium through sodium channels, as contributing to the cascade of Ca²⁺-induced axonal degeneration (Stys et al., 1992; Imaiizumi et al., 1997; Stys and Lopachin, 1998). Elevated levels of NO within multiple sclerosis lesions may contribute to this process by impairing mitochondrial function, thus disrupting ionic homeostasis (Kapoor et al., 2003). In support of an involvement of sodium channels and NCX in mechanisms leading to axon degeneration, Craner et al. (2004a) have reported the co-localization of sodium channel Na₆.1.6 and NCX in damaged axons in EAE and in acute multiple sclerosis plaques (Craner et al., 2004b), and Rush et al. (2005) have reported that persistent currents are carried by Na₆.1.6 channels. These observations are consistent with the suggestion that phenytoin exerts a protective effect on axons via a direct action on demyelinated and/or damaged axons, with blockade of sodium influx preventing inappropriate reverse operation of NCX and importation of injurious Ca²⁺.

It is also possible that a sodium channel blocker might indirectly provide protection for axons by its action on some immune cells. Bechtold et al. (2004) noted that flecainide reduced the severity of neurological symptoms early in chronic-relapsing EAE, and suggested that it might act via an immunomodulatory effect, e.g. by altering T-cell activation. Alternatively, sodium channel blockers might affect microglia and macrophages, which play multiple roles in neuroinflammatory process, including production of proinflammatory cytokines (Renno et al., 1995), phagocytosis of myelin (Li et al., 1996) and reactivation of T-cells (Jacobsen et al., 2002). The close proximity of macrophages to degenerating axons (Ferguson et al., 1997; Trapp et al., 1998; Kornek et al., 2000) and the release of macrophage- derived proteases (Anthony et al., 1998) and/or nitric oxide (Bolanos et al., 1997) also support a role for macrophages/microglia in the pathogenesis of axonal loss following demyelination. Electrophysiological studies have demonstrated the presence voltage-gated sodium channels in rat (Korotzer and Cotman 1992; Schmidtmaier et al., 1994) and human (Norenberg et al., 1994) microglia. Thus, blockade of sodium channels by phenytoin might be expected to alter microglia/macrophage activity and function in EAE. Consistent with this suggestion, an earlier study showed that phenytoin treatment reduced the inflammatory cell infiltrate within the spinal cord in C57/BL6 mice with EAE at 28–30 days post-EAE induction by 76% (Craner et al., 2005). In this study, we demonstrate a reduction of inflammatory cell infiltrate at both 90 and 180 days post-MOG injection in monophasic EAE and at 120 days post-injection in chronic-relapsing EAE. While we can not exclude the possibility that the reduction in CD45⁺ cells that we observed in phenytoin-treated EAE was related to a reduction in Wallerian-like axonal degeneration and a resulting reduction in microglial reactivity, it has been demonstrated that sodium channel Na₆.1.6 is upregulated in activated microglia in vitro and in vivo and that TTX can significantly inhibit the phagocytic activity of activated microglia in an in vitro model in which degenerating axons are not present (Craner et al., 2005). Thus, we propose that phenytoin may protect axons in EAE via two converging pathways, involving block of sodium channels on axons, and on microglia and/or macrophages. Because the use of the anti-CD45, which reacts with lymphocytes, monocytes and reactive microglial/macrophages, does not permit discrimination of phenytoin action on specific immune cell populations, future studies utilizing lymphocyte markers (e.g. anti-CD4, anti-CD8), and possibly focusing on areas where inflammation is greatest, will be needed to examine whether phenytoin also inhibits lymphocyte infiltration in EAE.

Our results carry several caveats. We would urge caution in extrapolating the observations of the present study in animal models to the use of phenytoin as a therapy for multiple sclerosis, and stress that the observations described here should not be viewed as guiding clinical practice. Moreover, as noted above, the mechanisms of action of phenytoin in EAE remain unresolved. Nonetheless, irrespective of the pathway(s) by which phenytoin provides protection of axons in monophasic and chronic-relapsing EAE, our results show that phenytoin treatment is effective both in monophasic and chronic-relapsing EAE models, and that it is effective both in the short-term and for extended periods of time, preventing loss of axons and enhancing clinical status. Thus, these results represent a step forward toward a controlled clinical study.
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