A combined inhibitor of matrix metalloproteinase activity and tumour necrosis factor-α processing attenuates experimental autoimmune neuritis

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Summary
Matrix metalloproteinases (MMPs) and the cytokine tumour necrosis factor (TNF)-α are implicated in the pathology of inflammatory demyelinating diseases of the CNS, and may also be involved in peripheral demyelinating diseases such as acute inflammatory demyelinating polyradiculoneuropathy. We have tested an inhibitor of MMP activity and TNF-α processing, BB-1101, in experimental autoimmune neuritis (EAN), an animal model of Guillain–Barre syndrome. Treatment with BB-1101 from the time of immunization prevented the development of EAN, and when given from the onset of symptoms, it significantly reduced disease severity. These results indicate that MMPs and/or TNF-α are involved in the pathogenesis of EAN, and that drugs of this type may have potential as novel therapeutic agents in the therapy of peripheral nervous system demyelinating diseases.

Keywords: demyelination; matrix metalloproteinases; tumour necrosis factor-α; experimental autoimmune neuritis; inflammation

Abbreviations: AIDP = acute inflammatory demyelinating polyradiculoneuropathy; CMAP = compound muscle action potential; EAE = experimental autoimmune encephalomyelitis; EAN = experimental autoimmune neuritis; ELISA = enzyme-linked immunosorbent assay; MMP = matrix metalloproteinase; PBS = phosphate-buffered saline; TNF = tumour necrosis factor

Introduction
Acute inflammatory demyelinating polyradiculoneuropathy (AIDP) is the most common pathological feature of Guillain–Barré syndrome (Asbury et al., 1969; Griffin et al., 1995). It causes acute paralysis in two per 100 000 of the population per year, requires artificial ventilation in a quarter of patients, has a mortality of 5–10%, and leaves 10% of survivors with persistent disability (Hughes, 1990). There is emerging evidence that matrix metalloproteinases (MMPs) may play a role in such inflammatory demyelinating diseases. MMPs are a group of zinc dependent enzymes which can degrade extracellular matrix components. They have been implicated in the breakdown of the blood–brain barrier (Rosenberg et al., 1992), and can degrade myelin basic protein (Chantry et al., 1989; Gijbels et al., 1993; Chandler et al., 1995) which is a major constituent of myelin in the CNS. Similar, or identical, converting enzymes are involved in the processing of the Fas-ligand (Mariani et al., 1995), vascular cell adhesion molecule 1 (Lecl et al., 1995), beta-2-microglobulin (Demaria et al., 1994), and receptors for nerve growth factor (DiStefano et al., 1993), interleukin-6 and tumour necrosis factor (TNF) (Mullberg et al., 1995). In addition, an MMP-like TNF converting enzyme is involved in the processing and release of the cytokine TNF-α (Gearing et al., 1994; McGeehan et al., 1994; Mohler et al., 1994).

MMPs have been implicated in the pathogenesis of inflammatory demyelinating diseases of the CNS such as multiple sclerosis. MMP-9 is elevated in the CSF of patients with multiple sclerosis (Gijbels et al., 1992), and is detected immunocytochemically in both active and chronic multiple sclerosis lesions (Gijbels and Steinman, 1994; Maeda and
Sobel, 1996). MMP-9 is also detected in the CSF of mice with experimental allergic encephalomyelitis (EAE) (Gijbels et al., 1993) and the severity of disease can be reduced by inhibitors of MMP activity (Gijbels et al., 1994; Hewson et al., 1995). In addition, combined inhibitors of MMP activity and TNF-α processing can reduce disease severity in EAE (Corkill et al., 1995). The pro-inflammatory cytokine TNF-α is also strongly implicated in the pathogenesis of multiple sclerosis and EAE (Rainé, 1995).

Despite this evidence of a role for MMPs in demyelinating diseases of the CNS, the involvement of MMPs in autoimmune demyelinating diseases of the PNS (peripheral nervous system) has not been investigated and forms the focus of the current study. Experimental autoimmune neuritis (EAN) is an accurate clinical, neurophysiological and histological model of AIDP, and can be induced in Lewis rats by immunization with bovine peripheral nerve myelin (Waksman et al., 1956; Kadlubowski et al., 1980; Harvey and Pollard, 1992a).

Synthetic hydroxamic acid-based drugs have been developed which inhibit MMP activity and also TNF-α release (Gearing et al., 1995; Beckett et al., 1996). In this study we have examined the efficacy of one such inhibitor, BB-1101, in the therapy of EAN.

Material and methods

Animals

Adult Lewis rats (male or female, 200–250 g, n = 136, Harlan, UK) were used in this study; each experimental group typically comprised 10 animals. They were housed in cages in groups of two to three and allowed free access to food and water throughout the study. Rats entered the study ≥5 days after arrival in the animal facility. The experimental procedures were conducted under a UK Home Office Licence.

Induction of EAN

Injections (0.05 ml, s.c.) of an emulsion (prepared according to the method of Kadlubowski et al., 1980) containing lyophilized bovine spinal root myelin (2.0 mg) and Mycobacterium tuberculosis (0.5 mg; Difco, Surrey, UK) in Freund’s adjuvant (Difco) were made in the right hind ankle) and nerve conduction velocity calculated. At the end of each experiment all the rats were anaesthetized (1.5–2% halothane in oxygen), and the rectal and subcutaneous left leg temperatures were monitored and maintained at 37°C. The sciatic and tibial nerves of the left leg were stimulated with supramaximal electrical stimuli delivered at the sciatic notch and ankle, respectively, using needle electrodes (1 Hz, pulse width 0.1 ms). EMG activity evoked by the stimulation was recorded from the dorsal surface of the foot. Records were averaged on a digital oscilloscope (Gould DSO 420) and stored on computer. The amplitude and latency of the compound muscle action potential (CMAP) following stimulation at the sciatic notch or ankle were measured and the CMAP amplitude ratio (sciatic : ankle) and nerve conduction velocity calculated.

Clinical observation and scoring of disease severity

Animals were weighed daily and examined for the presence of any clinical deficit by an observer blinded as to the treatment regimen. A scoring system was used in which one point of clinical deficit was awarded to each animal for each of the following signs: 5% weight loss in 2 days; animal looked ill; animal appeared hunched; decreased tone of tail tip; decreased tone of whole tail; tail paralysis; decreased toe spreading; animal walked with a sprawling gait; slow righting reflex; no righting reflex; dragging of one hind limb; dragging of two hind limbs; forelimb weakness; paralysis of one limb; paralysis of two limbs; paralysis of three limbs; animal appears moribund; animal humanely sacrificed. The maximum daily clinical score which could be attained was 18. This scoring system was shown to have a satisfactory inter-ratative reliability, since the intraclass correlation between the scores assigned by different observers to the same animals was high (r = 0.96).

EMG recording

EMG recordings were made from selected animals at the end of the study period. Rats were anaesthetized (1.5–2% halothane in oxygen), and the rectal and subcutaneous left leg temperatures were monitored and maintained at 37°C. The sciatic and tibial nerves of the left leg were stimulated with supramaximal electrical stimuli delivered at the sciatic notch and ankle, respectively, using needle electrodes (1 Hz, pulse width 0.1 ms). EMG activity evoked by the stimulation was recorded from the dorsal surface of the foot. Records were averaged on a digital oscilloscope (Gould DSO 420) and stored on computer. The amplitude and latency of the compound muscle action potential (CMAP) following stimulation at the sciatic notch or ankle were measured and the CMAP amplitude ratio (sciatic : ankle) and nerve conduction velocity calculated.

Collection of tissue

At the end of each experiment all the rats were anaesthetized with 1.5–2% halothane in oxygen and tissue taken for histological examination. In selected animals the right sciatic nerve was removed and either snap-frozen in liquid nitrogen for use in zymography, or embedded in OCT (Tissue Tek, Miles Diagnostics, Elkhart, Ind., USA) and frozen using solid CO2 to assess the infiltration of T cells and macrophages into the nerve by immunocytochemistry (see below). Blood samples (2–5 ml) were taken by cardiac puncture to determine whether anti-myelin antibodies were present. Some animals underwent cardiac perfusion with warmed (37°C) saline (0.9% sodium chloride containing 2 IU/ml heparin, 0.05% lignocaine, 0.05% sodium nitrite, 0.01 M HEPES, pH 7.4) followed by 3% glutaraldehyde (in 0.1 M phosphate buffer) and the cauda equina and left sciatic nerve removed. In the remaining animals the left sciatic nerve was removed and fixed by immersion in 3% glutaraldehyde before collection of the blood sample. The glutaraldehyde-fixed tissues were processed into resin using a standard protocol (Redford et al., 1995). Transverse semi-
thin (1-µm) sections were stained with thionin acetate and acridine orange for examination at light microscopy.

**Immunocytochemistry protocol**

Transverse sections of the frozen nerves were cut at 10 µm using a cryostat (Bright Instruments), and picked up on gelatinized slides. Sections were fixed for 10 min in absolute alcohol (4°C), and preabsorbed with 10% normal horse serum. The binding of primary antibodies was revealed by an avidin–biotin–peroxidase method (Hsu et al., 1981) (Vector Labs, UK). Staining of nerves from different groups was carried out at the same time to reduce interassay variation. The primary antibodies used in this study were ED-1 (1 : 300; Serotec, UK) which identifies macrophages, OX-19 (MRC Cellular Immunology Unit, Oxford, UK) which identifies CD5+ positive T-lymphocytes and SE 601, a polyclonal antibody raised in rabbit against a synthetic peptide sequence specific for 92-kDa gelatinase. A monoclonal antibody specific for 92-kDa gelatinase, 2C10, was also used and showed identical immunostaining to SE 601 (data not shown). Biotinylated rat absorbed anti-mouse IgG (1 : 100) and anti-rabbit IgG (1 : 200, Vector Labs) were used as the secondary antibodies. Control sections were treated in the same way, omitting incubation with the primary antibody. SE 601 and 2C10 were also preabsorbed against the peptide, all showed no staining.

A total of 10 areas, each 0.03 mm², which covered a distance of 2 mm, were analysed for each nerve (nine rats in each group) under high magnification. The numbers of macrophages and T cells were assessed 21 days after inoculation with bovine myelin and were expressed as the number of cells per square millimetre of nerve tissue.

**Zymography**

Gelatinase activity in sciatic nerves was assayed by zymography as described previously (Rosenberg et al., 1994), with modifications. Frozen nerves from control animals and from rats 21 days post inoculum were flattened in liquid nitrogen using a stainless steel pestle and mortar. The tissue was then weighed and lysis buffer (20 mM PBS, 0.05% Triton X-100 and a cocktail of proteinase inhibitors; Sigma, UK) added to give a final concentration of 200 µg/ml. The tissue was homogenized in a Dounce homogenizer, left overnight (4°C), spun (13 000 r.p.m., for 15 min) to pellet the debris and the supernatant was frozen in aliquots at −70°C. Each sample was diluted in lysis buffer to 40 µg/ml. Then, 40 µl of each sample was run under denaturing, but non-reducing, conditions through precast 10% polyacrylamide minigels containing 0.1% gelatin (Novex). The gels were then incubated for 1 h at room temperature in a 2.5% Triton X-100 solution, rinsed with water and then incubated for 24 h at 37°C in a low salt buffer (50 mM Tris–HCL, pH 7, 200 mM NaCl, 5 mM CaCl₂, 0.02% Brij-35). The gels were stained with 0.5% Coomassie blue in 40% methanol and 10% acetic acid, revealing zones of clearance which corresponded to enzymatic activity. A control zymogram to confirm specificity of MMP activity was prepared simultaneously by adding the broad spectrum MMP inhibitor, BB-2116 (10 µM) to the incubating solutions. Human 72-kDa gelatinase (5 ng) and 92-kDa gelatinase (1 ng) were used as standards and marker proteins (Novex) were used to estimate molecular weight.

**Determination of anti-myelin antibodies**

Anti-peripheral nerve antibodies were determined by enzyme-linked immunosorbent assay (ELISA) using myelin isolated from either bovine or rat cauda equina as antigen. The method of coating the ELISA plate was adapted from the method of Challacombe et al. (1983). Myelin suspensions were diluted to a protein concentration of 50 µg/ml in 0.3% methylglyoxal, freshly prepared in water from a 2% stock solution (prepared by diluting a stock 40% solution in water and adjusting the pH to 8.0 with sodium bicarbonate). Then, 50 µl of the diluted myelin suspension was added to each well and the plates incubated at 37°C for 2 h. The wells were emptied and washed carefully three times with 250 µl of PBS before blocking with 50% foetal calf serum in PBS, 150 µl per well, for 2 h at 37°C. After washing once with PBS, 50 µl of rat serum diluted 1/250 in 5% foetal calf serum in PBS, was added and incubated overnight at 10°C. After washing three times, alkaline phosphatase conjugated goat anti-rat-IgG (whole molecule) (Sigma), 50 µl per well at 1/1000 dilution, was added for 1 h at 37°C. The plates were then washed three times with PBS, and developed with p-nitrophenyl phosphate for 30 min at 37°C, and the optical density read at 405 µm. The mean of quadruplicate assays was calculated. As whole myelin was used as the target antigen in the ELISA, and since this is a complex ‘antigen’ containing many different proteins, anti-myelin antibody levels are reported as the absorbances at a single serum dilution.

**Results**

**Preventative effect of BB-1101 treatment**

The development of EAN was prevented in animals which received BB-1101 (either 2 mg/kg, i.p., twice daily, or 10 mg/kg, s.c., once daily) from the day of immunization with bovine myelin. These treatments prevented both the development of neurological deficits (Fig. 1A) and the weight loss (Fig. 1B). In contrast, weight loss and increasing muscle weakness began 11 days after immunization with myelin in control animals which received injections of vehicle (PBS–Tween) (Fig. 1A and B). In these control animals alone, disease progressed to reach peak severity.
by day 16, after which the animals gradually recovered (Fig. 1A).

EMG recordings made at the end of the experiment from animals treated with BB-1101 revealed no conduction abnormalities when compared with normal animals [mean CMAP amplitude ratio (sciatic : ankle) for animals receiving BB-1101 was 0.87 ± 0.02, whilst the range of CMAP amplitude ratio for normal animals was 0.78–0.90 (our unpublished observations)]. In contrast, control animals immunized for EAN, but given daily injections of vehicle alone, showed a marked reduction in CMAP amplitude ratio (mean CMAP amplitude ratio was 0.70 ± 0.06, \( P < 0.05 \) compared with BB-1101 + EAN animals, unpaired \( t \) test) indicating the presence of conduction block in some axons. Consistent with these electrophysiological findings, histological examination of sciatic nerves in 1-\( \mu \)m resin sections showed that marked endoneurial inflammation and numerous demyelinated and degenerated axons were prominent in the nerves of vehicle-injected animals (Fig. 2B). In animals which had received BB-1101, the sciatic nerves appeared relatively normal (Fig. 2C). Semi-quantitative assessment of the extent of demyelination and axonal degeneration in sciatic nerves, indicated that BB-1101 reduced these pathologies (Fig. 2, lower figures).

Many CD5+ T-lymphocytes and ED-1 positive macrophages with a ‘foamy’ phagocytic appearance were detected immunocytochemically in the sciatic nerves of animals which had received daily injections of vehicle (Fig. 3B and F). The extent of cellular infiltration by these cells increased in parallel with the increase in clinical score (data not shown). In marked contrast, BB-1101 treatment prevented the infiltration of CD5+ T-lymphocytes and macrophages into the sciatic nerves (Fig. 3).

Immunization with bovine cauda equina induced the production of antibodies directed against both the inoculum, bovine peripheral nerve myelin, and cross-reactive auto-antibodies against rat peripheral nerve myelin. Western blot analysis against rat peripheral nerve myelin indicated that antibodies were directed against multiple protein antigens, with antibodies against the major myelin protein \( P_0 \) the most prominent. Administration of BB-1101 did not affect the anti-myelin antibody level irrespective of how the drug was administered (Fig. 4). This observation indicates that BB-1101 did not interfere with the humoral immune response.

**Administration of BB-1101 from day 7 after immunization**

A significant reduction in clinical score (Fig. 5A) was seen from day 17 after immunization when BB-1101 (10 mg/kg, in PBS–Tween, s.c.) had been administered once daily from day 7 after immunization. There was a small reduction in the loss in body weight, but it did not attain statistical significance (Fig. 5B).

**Therapeutic effect of BB-1101**

In experiments where BB-1101 was given from the onset of symptoms, the clinically relevant regimen, rats with a similar severity of symptoms (e.g. a decrease of 5% body weight over 2 consecutive days) were allocated in pairs to receive either BB-1101 (10 mg/kg, s.c., twice daily, prepared in DMSO-PBS–Tween) or vehicle (DMSO-PBS-Tween) from that point on. EAN continued to develop in both groups, but animals treated with BB-1101 showed a slower progression of disease, and a markedly reduced peak clinical score (Fig. 5C). This treatment did not significantly affect the weight loss observed (Fig. 5D).

**Detection and localization of 92-kDa gelatinase by immunohistochemistry**

Both the polyclonal antibody SE 601 and the monoclonal antibody 2C10 (data not shown) showed the same pattern of
Fig. 2 The upper figures show 1-μm transverse resin sections through the sciatic nerves of (A) a normal, unimmunized rat, (B) a rat with EAN which had received daily injections of vehicle (PBS-Tween) from the time of immunization with myelin 21 days previously and (C) a rat which had received BB-1101 daily from the time of immunization with myelin 21 days previously (all at ×960 magnification). The lower figures show the median results of a semi-quantitative assessment of the sciatic nerves from individual rats for the presence of demyelination and axonal degeneration depending on whether the nerve was unaffected (0), mildly (1), moderately (2) or severely affected (3). In normal rats (A, upper and lower figure), no demyelination or axonal degeneration were present. The sciatic nerves of rats with EAN which had received daily injections of vehicle (B, upper figure) were markedly different from those of normal rats. An inflammatory response was still evident within the nerve, including myelin-debris containing macrophages (m). Numerous demyelinated axons (d), and axons which have undergone Wallerian degeneration (w) were present within the nerve at this time. Semi-quantitative assessment of the nerves (B, lower figure) indicated that both demyelination and axonal degeneration were present. When BB-1101 was given daily from the time of immunization with myelin, the sciatic nerve appeared normal (C, upper figure) and was similar to the sciatic nerves from normal rats (A, upper figure). Treatment with BB-1101 significantly reduced the amount of demyelination present in the sciatic nerves of myelin-immunized animals (C, lower figure). *P < 0.05 compared with vehicle-injected animals, Kruskal–Wallis one-way ANOVA. The resin sections were stained with thionin acetate and acridine orange.
staining for 92-kDa gelatinase. The enzyme was localized to the Schwann cells and blood vessels in normal sciatic nerve (Fig. 6A and C). The expression in Schwann cells remained the same, 21 days post inoculum, but immunostaining around the endoneurial blood vessels was more intense. Infiltrating T-lymphocytes cuffed endoneurial blood vessels were also positive for 92-kDa gelatinase (Fig. 6B and D), as identified in consecutive sections labelled with OX-19 (not shown).

Gelatinase activity increased in EAN sciatic nerves

In naive control sciatic nerves, 72-kDa gelatinase activity was detected, as was a less prominent band with a molecular weight of ~62 kDa (lane 3, Fig. 7). This zone of clearance was due to MMP activity and is likely to be attributable to an activated form of 72-kDa gelatinase. The activity at both these molecular weights was increased in EAN; in addition, activity was identified at 92 kDa (lane 4, Fig. 7). Confirmation of MMP specificity was obtained by addition of BB-2116 (10 μM), a broad spectrum MMP inhibitor, during the development of the gels. BB-2116 abolished all the gelatinase activity (results not shown).

Discussion

We have shown that BB-1101, an inhibitor of both MMP activity and TNF-α processing (Corkill et al., 1995), was effective in preventing the development of EAN when given from the day of immunization. Endoneurial inflammation and demyelination were both significantly reduced in treated animals. Furthermore, disease severity in EAN was reduced when BB-1101 was administered either from day 7 after immunization with myelin, or from the onset of neurological symptoms.

The severity of EAN can be reduced by a number of therapeutic approaches including suppression of the immune response with agents such as cyclosporin (Nakayasu et al., 1990), attenuation of the inflammatory response using steroids (Watts et al., 1989) or non-steroidal anti-inflammatory agents (Hartung et al., 1988), or by using compounds directed against mediators released during EAN (Stoll et al., 1993; Maeda et al., 1994). However, this is the first study to demonstrate the efficacy of an MMP inhibitor in the treatment of EAN.

The findings with BB-1101 have allowed us to gain further insight into the aetiology of EAN, which may also be relevant to AIDP. EAN is an autoimmune disease, whose initiation is considered to be dependent on both the development of an antibody response against myelin proteins, and the production of activated T cells specific for nerve antigen(s) (Hartung et al., 1995). The presence of an antibody response is not sufficient to cause EAN, since the animals which received BB-1101 from the time of immunization did not develop EAN, even though they developed an anti-myelin antibody response which was of similar magnitude to that seen in vehicle-treated animals. In support of this finding, EAN is not induced in naive animals by the passive transfer of serum from animals with EAN (Harvey and Pollard, 1992b).

The effects of BB-1101 in EAN may be explained in terms of its inhibitory effect on MMPs, and by the potential of MMPs to influence several aspects of the inflammatory process. MMPs are secreted by activated leukocytes in culture (Hibbs et al., 1987; Montgomery et al., 1993; Romanic and Madri, 1994), and degrade components of the extracellular matrix found in the basal lamina (Woessner, 1993). In order for inflammatory cells to enter the tissue from the blood, they must cross the basal lamina surrounding blood vessels. MMPs increase vascular permeability in the CNS, opening the blood–brain barrier (Rosenberg et al., 1992) and, although it has not yet been determined, it is likely that MMPs exert
Fig. 5 A and C show the progression in median clinical score, and B and D the change in body weight, of rats immunized with an emulsion containing bovine myelin at day 0. The duration of treatment with either BB-1101 (10 mg/kg, s.c.; filled squares) or vehicle (open diamonds) is shown by the shaded bar. Administration of BB-1101 (in PBS–Tween) from day 7 after immunization significantly reduced disease severity (A), but did not affect the weight loss observed (B). Treatment with BB-1101 (in DMSO–PBS–Tween) from symptom onset also significantly reduced disease severity (C), without significantly affecting the weight loss observed (D). Values in B and D are means ± SEM. *P < 0.05, Mann–Whitney U test.
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Effects due to the inhibition of processing of other cytokines, growth factors and adhesion molecules may also contribute to the efficacy of BB-1101.

The neurological deficit in EAN is a consequence of conduction block in demyelinated axons and axonal degeneration in the PNS (Kadlubowski et al., 1980; Harvey and Pollard, 1992a). Inflammation and/or opening of the blood–nerve barrier may be necessary for the pathological changes in nerve fibres in EAN. These changes develop in response to the passive transfer of serum from animals with EAN only when endoneurial inflammation is present (Harvey et al., 1995), or when the blood–nerve barrier has been opened (Spies et al., 1995). BB-1101, by preventing the endoneurial inflammatory response, may reduce nerve fibre damage and thereby the neurological deficit when given from the day of immunization.

The finding that treatment with BB-1101 from symptom onset did not prevent the development of EAN, but rather reduced disease severity was not unexpected. When symptoms are evident, inflammation is already present within the PNS (Kadlubowski et al., 1980), therefore BB-1101 may only inhibit the progression and maintenance of the inflammatory response when given from this time. Steroids (Watts et al., 1989), or compounds which inhibit the release (Maeda et al., 1994) or action (Stoll et al., 1993) of TNF-α also reduce disease severity when given from symptom onset.

Since BB-1101 is beneficial in EAN, MMP inhibitors of this type have potential as therapeutic agents in AIDP and chronic inflammatory demyelinating polyradiculoneuropathy. The existing regimens which are of value in Guillain–Barré syndrome include plasma exchange and intravenous immunoglobulin, but even with these therapies recovery is slow and only 70% of patients have recovered 1 year later (The Italian Guillain–Barré Study Group, 1996). The addition of an MMP inhibitor treatment to either regimen may shorten the disease duration and reduce the residual deficit by diminishing the inflammatory process in demyelinating diseases.
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References


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