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Suppression of experimental allergic encephalomyelitis in the Lewis rat by the matrix metalloproteinase inhibitor Ro31-9790

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Abstract. Matrix metalloproteinases (MMPs) are implicated in the tissue destruction associated with inflammatory demyelinating diseases such as multiple sclerosis. The effect of a hydroxamate inhibitor of MMPs, Ro31-9790, on inflammatory demyelination was assessed in two acute models of experimental allergic encephalomyelitis (EAE). Daily intraperitoneal injections of Ro31-9790 (50 mg kg^{-1}), beginning either at the time of disease induction or from day 3 post induction, significantly reduced the clinical severity of adoptively transferred EAE. Administration of the inhibitor from the day of induction of active EAE prevented disease onset in 9/10 animals. However, in a repeat study, in which clinical disease was much more severe in the vehicle treated animals, the inhibitor was less effective. Clinical signs and CNS histopathology correlated well, with greater numbers of inflammatory lesions associated with increased disease severity. The present study confirms a role for the MMP cascade in inflammation in EAE.

Key words: Matrix metalloproteinase inhibitor – Experimental allergic encephalomyelitis – Multiple sclerosis – Inflammation

Introduction

Blood-brain barrier (BBB) breakdown and inflammatory cell infiltrates accompanied by limited demyelination in the central nervous system (CNS) are pathologic characteristics of experimental allergic encephalomyelitis (EAE), an animal model of the human demyelinating disease multiple sclerosis (MS) [1].

Proteolytic enzymes are among agents with the potential to permeabilize the BBB [2] permitting the extravasation of inflammatory cells from the circulation through the cerebral endothelium into the CNS. Involvement of matrix metalloproteinases (MMPs) in the physiological and pathological breakdown of connective tissue has been indicated not only by their release from cells present at the site of tissue destruction, in particular those of the monocyte/macrophage lineage, but also because their release and activation is stimulated by inflammatory cytokines such as interleukin (IL)-1 [3], tumour necrosis factor (TNF)-α [4], IL-8 [5] and by direct cell-cell contact [6]. Evidence for neutral protease involvement in myelin breakdown is also well documented [7–10]. More recently, one member of the family of MMPs, gelatinase-B (MMP-9, 92 kDa) has been shown to cleave human and guinea-pig myelin basic protein (MBP) into fragments which correspond to encephalitogenic epitopes for inducing EAE in mice and guinea-pigs [11, 12]. Gelatinase B, in contrast to the constitutively synthesised gelatinase A (MMP-2, 72 kDa), is present in the cerebrospinal fluid (CSF) of patients with inflammatory neurological disorders such as MS [13] and in the CSF of mice with EAE [11]. It has also been demonstrated in MS lesions, both by immunocytochemistry [14] and by mRNA in situ hybridisation [unpublished observation].

Previous studies have demonstrated the ability of serine, carboxy and thiol inhibitors of proteases to suppress the development of EAE [15–18]. In this study we have used a potent hydroxamate inhibitor of gelatinase B (Ro31-9790) to assess the role of MMPs in both actively induced and adoptively transferred EAE, on the basis that it could modulate the entry of primed lymphocytes and the demyelinating potential of macrophages.

Methods

Animals

Female Lewis rats (190–220 g) were purchased from Charles River, Kent, U.K. and housed in pairs in a standard animal facility. All animals were allowed free access to food and water.

Induction of active EAE (act-EAE)

Animals were injected in each hind footpad with 50 µg of guinea-pig MBP (final concentration 1 mg ml^{-1}), prepared by the method of

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Dunkley and Carnegie [19], emulsified in complete Freund's adjuvant (MBP-CFA) containing heat killed *Mycobacterium tuberculosis* H37Ra (final concentration 5 mg ml^{-1}; Difco Laboratories, U.K.).

Adoptive transfer of EAE (AT-EAE)

Eleven days after MBP-CFA immunisation, animals were sacrificed and the spleens removed. Splenic lymphocytes were cultured at 2×10^6 cells ml^{-1} in RPMI 1640 medium supplemented with 10% heat inactivated foetal calf serum, 2 mM glutamine, 100 U ml^{-1} penicillin, 100 µg ml^{-1} streptomycin (Gibco Ltd., Paisley, Scotland), 2 µM 2-mercaptoethanol, 1 mg ml^{-1} indomethacin (Sigma, Poole, U.K.) with MBP at 10 µg ml^{-1} for 72 hrs at 37 °C in an atmosphere of 5% CO_2 : 95% O_2. Harvested cells were washed thoroughly in unsupplemented RPMI 1640 and 4×10^7 splenocytes were transferred intraperitonneally to naive recipients.

Assessment of clinical EAE

Animals were weighed daily and observed for clinical signs of EAE which were scored as follows: (0) no clinical signs; (1) flaccid tail and weight loss; (2) hind limb hypotonia with further weight loss; (3) complete hind limb paralysis. In addition, intermediate scores were assigned to animals which, for example showed a loss of tonicity in the distal half of the tail (score = 0.5) or paralysis of one hind limb (score = 2.5).

Metalloproteinase inhibitor and treatment protocol

Ro31-9790 (Roche Products Limited, Welwyn Garden City, Herts, U.K.) was dissolved in 4% gelofusine (Consolidated Chemicals, U.K.) by wet milling to a concentration of 25 mg ml^{-1}. Ro31-9790 shows IC50 values of 5, 12 and 470 nM for collagenase, gelatinase B and stromelysin respectively [20].

For act-EAE, treatment began either on day 0 prior to EAE induction or on day 10 following induction. For AT-EAE, treatment began either on day 0 prior to cell transfer or on day 3 post transfer. Ro31-9790 was administered daily by intraperitoneal injection at a dose of 50 mg kg^{-1}. Control animals received vehicle (0.4 ml, 4% gelofusine) from day 0 until the end of the experiment. Plasma levels of Ro31-9790, measured by HPLC-MS at Roche, Welwyn Garden City, confirmed the reproducibility of the drug administration protocol.

Assessment of histological EAE

Spinal cords were removed from animals following recovery from EAE, i.e. day 10 post-induction for AT-EAE and day 18 post-induction for act-EAE, and were rapidly frozen on solid CO_2. Longitudinal frozen sections (approx 1.5 cm in length; 10 µm thick) of brain stem/cervical spinal cord were cut on a cryostat (Slee, U.K.) and stained with haematoxylin and eosin. Histopathological scores were calculated as follows: individual lesions were scored as 1 = perivascular inflammation ≤ three cells thick; 2 = >three cells thick; 3 = parenchymal infiltrate. The histopathological score was then calculated for each animal by adding all the scores for individual lesions in a section and the mean of 2–3 individual sections taken.

Statistical analysis

Differences between vehicle and Ro31-9790 treatments on clinical and histopathological scores were assessed using the Mann–Whitney U-test. Significance was accepted if $p < 0.05$.

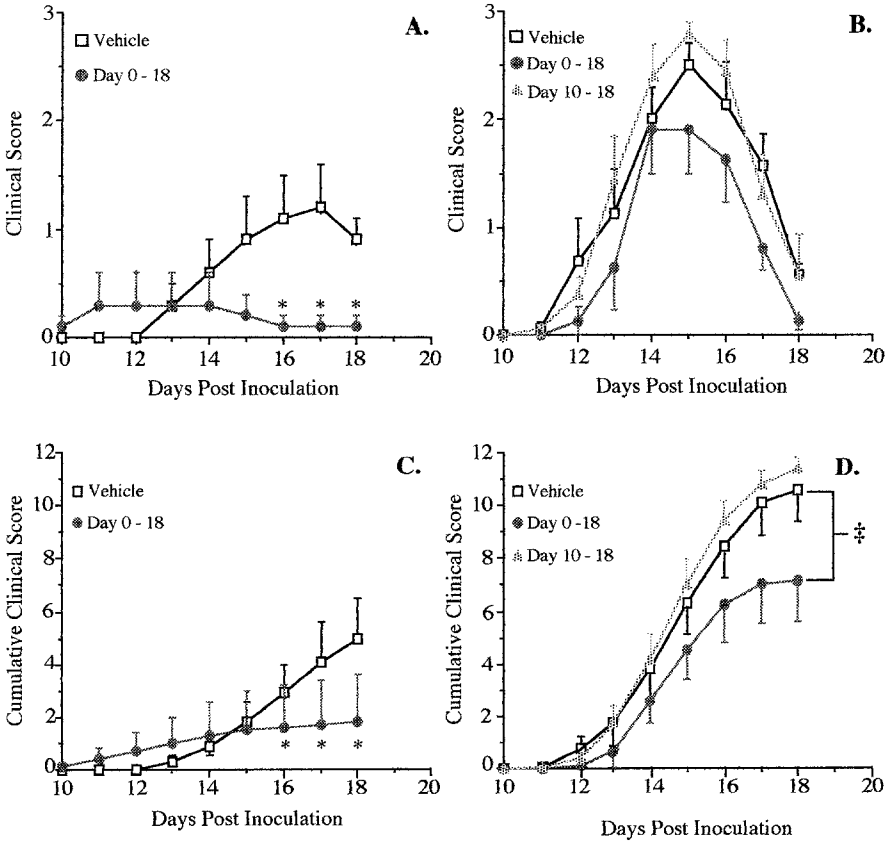

Fig. 1. Clinical (A, B) and cumulative clinical (C, D) scores of female Lewis rats administered either vehicle (4% gelofusine i.p., day 0 to day 18) or Ro31-9790 (50 mg/kg i.p.) as either a prophylactic (day 0 to day 18) or therapeutic (day 10 to day 18) regime during the course of active EAE. (A, C: $n = 10$/group, Expt. 1; B, D: $n = 8$/group, Expt. 2). Points represent mean and sem. *$p \leq 0.05$ compared to vehicle treated animals; ‡$p = 0.025$ Ro31-9790 (day 0–18) compared to vehicle treated animals on day 18, Man–Whitney U-test.

Table 1. Effect of Ro31-9790 on active and adoptive transfer EAE in Lewis rats.

Treatment	Incidence	Onset[a]	Severity[b]
Active EAE			
Expt. 1			
Vehicle	9/10	15 ± 0.7	1.5 (0–3)
Ro31-9790 (day 0–18)	1/10	10	0 (0–3)[c]
Expt. 2			
Vehicle	8/8	12.9 ± 0.4	3 (1–3)
Ro31-9790 (day 0–18)	8/8	13.4 ± 0.3	2.5 (0.5–3)
Ro31-9790 (day 10–18)	8/8	12.6 ± 0.4	3 (3–3)
Transfer EAE			
Vehicle	6/6	5 ± 0	3 (2–3)
Ro31-9790 (day 0–10)	4/6	5.5 ± 0.3	1.5 (0–3)
Ro31-9790 (day 3–10)	5/6	5.2 ± 0.2	2 (0–3)

[a] Mean day of onset for the group ± s.e.m.
[b] Median (range) of maximum clinical score of individual animals.
[c] $p = 0.01$ compared with vehicle treated animals (Mann–Whitney U-test).

Results

Effect of Ro31-9790 on actively induced EAE

Figure 1 shows the clinical course of act-EAE in two separate experiments. In the first experiment a mild disease course was observed in vehicle treated animals and the mean clinical score was significantly reduced on days 16–18 in animals treated with Ro31-9790 from the day of disease induction (day 0) (Figure 1A). Furthermore, Ro31-9790 prevented the development of EAE in 9 out of 10 animals whereas clinical signs of EAE were apparent in 9 of the 10 vehicle treated animals; maximum clinical signs were also significantly less in the Ro31-9790 group ($p = 0.01$; Table 1, Expt. 1). In a second experiment the disease course was more severe in the vehicle treated group and although the mean clinical score was reduced in animals administered Ro31-9790 from day 0, this was not significant on any individual day (Figure 1B). Similarly, the maximum clinical signs were reduced in those treated with Ro31-9790 from day 0, although this did not achieve significance ($p = 0.052$; Table 1, Expt. 2). However, when expressed as the mean cumulative clinical score a significant reduction in disease severity was observed in the group treated with Ro31-9790 from day 0 ($p = 0.025$, day 18; Figure 1D). Animals treated with Ro31-9790 from day 10 displayed signs of EAE which closely followed those of vehicle treated animals (Figures 1B and 1D).

Effect of Ro31-9790 on adoptive transfer EAE

Figure 2A shows the clinical scores of animals treated with either vehicle or Ro31-9790 from day 0 or day 3 following the transfer of 4×10^7 MBP-sensitized splenocytes. Treatment with Ro31-9790 either from day 0 or from day 3 following cell transfer significantly reduced clinical disease severity on day 5 ($p = 0.001$; Figure 2A). Disease severity remained significantly reduced in animals treated with Ro31-9790 from day 0 on day 6 ($p = 0.01$) after which time all animals began to recover (Figure 2A).

Ro31-9790 completely prevented the development of clinical signs in two out of six animals treated from day 0 and in one out of six treated from day 3 (Table 1). The decrease in clinical disease severity following treatment with Ro31-9790 is further reflected in the significant decrease in the mean cumulative clinical score in both drug treated groups from the onset of clinical signs on day five ($p < 0.05$; Figure 2B).

Effect of Ro31-9790 on CNS inflammation

In experiment 1 CNS inflammation was assessed in four animals from each group. In the vehicle treated group, the animals which developed clinical scores of 3 had histopathological scores of 36 and 38 whereas two animals which had shown maximum clinical signs of 1 had histopathological scores of 3 and 5. In the Ro31-9790 treated animals, no inflammation was seen in those which were free of clinical signs and a histopathological score of 17 was seen in the animal which displayed a clinical score of 3. The difference between the two groups was not significant ($p = 0.057$; Figure 3A).

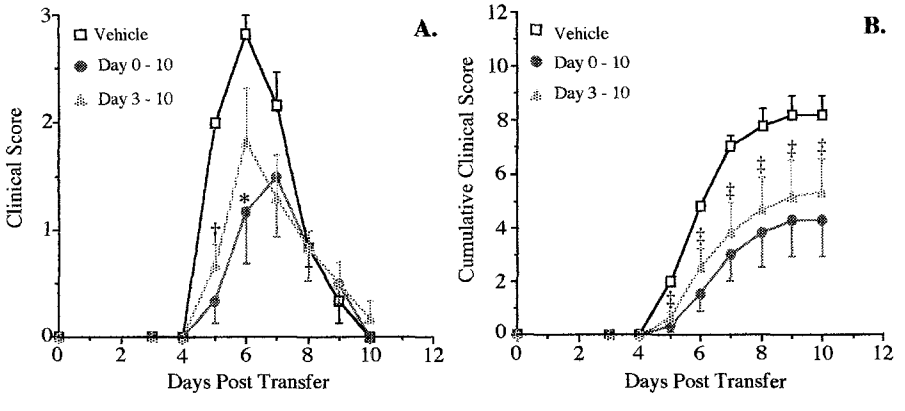

Fig. 2. (A) Clinical scores and (B) cumulative clinical scores of Lewis rats administered either vehicle (4% gelofusine i.p., day 0 to day 10) or Ro31-9790 (50 mg/kg i.p.) as either a prophylactic (day 0 to day 10) or therapeutic (day 3 to day 10) regime following the transfer of 4×10^7 MBP-sensitized splenocytes ($n = 6$/group). Points represent mean and sem. †$p = 0.001$ both Ro31-9790 treated groups compared to vehicle treated animals; *$p = 0.01$ Ro31-9790 (day 0–10) compared to vehicle treated animals; ‡$p < 0.05$, both Ro31-9790 treated groups compared to vehicle treated animals, Mann–Whitney U-test.

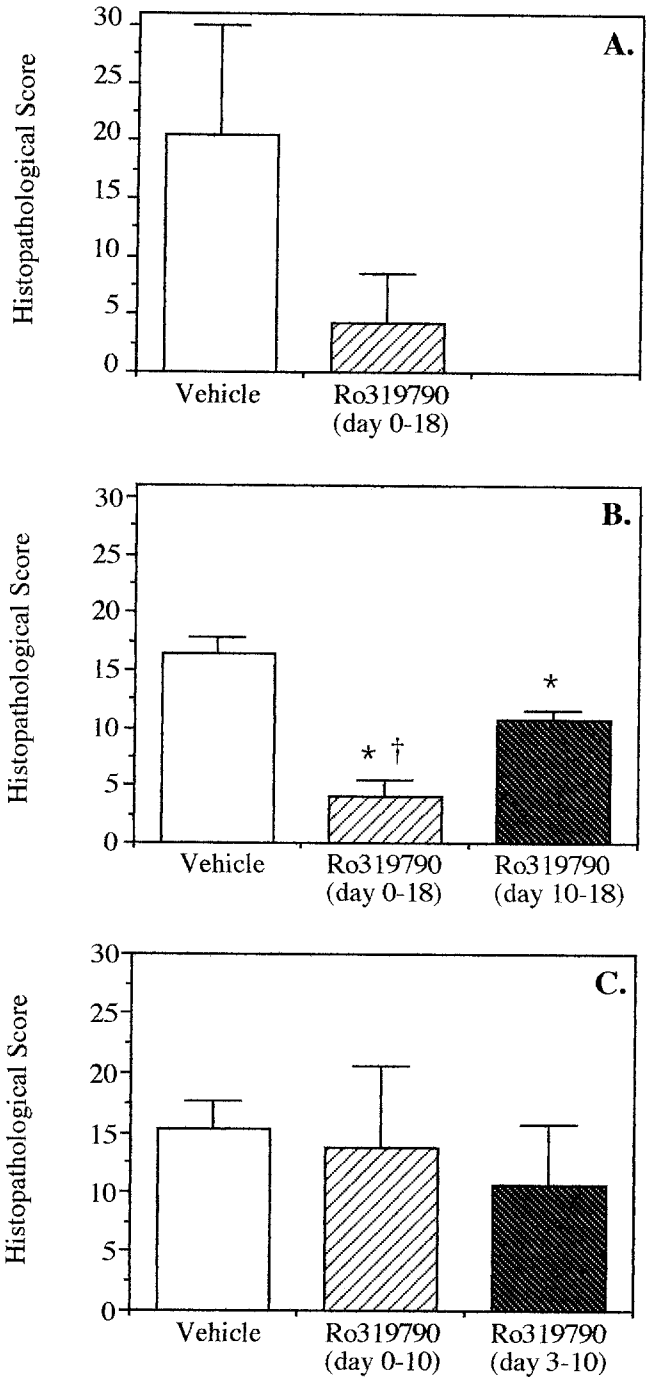

Fig. 3. Histopathological scores, calculated as described in Methods, for Lewis rats following recovery from active EAE (A, Expt. 1 [$n = 4$/group] and B, Expt. 2 [$n = 8$/group]) or adoptive transfer EAE (C [$n = 6$/group]). *$p \leq 0.002$ compared to vehicle treated animals; †$p \leq 0.002$ compared to Ro31-9790 (day 10–18) treated animals, Mann–Whitney U-test.

In experiment 2 all eight animals from each treatment group were examined histologically. Treatment with Ro31-9790 either from day 0 or from day 10 resulted in a significant reduction in histopathological score ($p \leq 0.002$ compared to vehicle treated animals; Figure 3B). This effect was most marked in those animals treated from day 0, with 4 animals from this group having only 0 to 2 inflammatory lesions and maximum clinical scores of 0.5, 0.5, 1 and 3.

In the AT-EAE model all six animals from each group were assessed for inflammatory lesions. No differences were found between vehicle and Ro31-9790 treated animals (Figure 3C). However, three animals treated with Ro31-9790 from day 0 had histopathological scores of 0, 0 and 3 corresponding to maximum clinical scores of 0, 0 and 1. A similar pattern was seen in animals treated from day 3.

Discussion

This study has shown that administration of the hydroxamate MMP inhibitor Ro31-9790 reduces the severity of EAE induced both by primary sensitization and following transfer of MBP-primed splenocytes. In the actively induced model the beneficial effect was greatest in animals with moderate clinical signs, declining in those with more severe symptoms and in both situations there was a good correlation between clinical severity and histological pathology.

Our data largely confirms that of Gijbels et al [21] who have recently demonstrated the suppression of active EAE in SJL/J mice upon treatment with another hydroxamate MMP inhibitor, commencing either at the time of disease induction or following the onset of clinical signs. Similarly, treatment with D-penicillamine which inhibits gelatinase B directly and indirectly by preventing the activation of progelatinase B by plasminogen activators, suppressed the development of EAE in SJL/J mice and attenuated the recurrence of disease in chronic relapsing EAE in Biozzi mice (G. Opdenakker, personal communication).

In the present study, prophylactic administration of the inhibitor was effective in ameliorating both adoptively transferred and active EAE. However, a therapeutic dosing regime was effective only in modulating the clinical course of adoptively transferred EAE. It is clear from this and the differing levels of inhibition found in the two active EAE experiments that the intensity of the immunological insult has a profound influence. In the first experiment, in which Ro31-9790 was 90% effective in preventing disease onset, the mean cumulative clinical score in the vehicle treated control group was half that observed in the second experiment. In the latter the severe clinical symptoms were less well controlled by the circulating levels of the drug although the cumulative clinical score was measurably reduced.

MMP inhibitors might be expected to work at two levels – by blocking the extravasation to the CNS and effector properties of lymphocytes and macrophages in the initial stage of inflammation and by limiting the myelin loss in the established lesion. More specifically, the inhibitors appear to prevent degradation of extracellular matrix and basement membrane but have no influence on the priming of MBP-specific T cells. This is supported by the results of Gijbels et al [21] who found in their study that the course of clinical disease became essentially the same in inhibitor-treated as in vehicle-treated animals after cessation of treatment.

In both animal groups with actively induced EAE there was a significant reduction in inflammation in the treated animals indicating that the inhibitor is predictably retarding leukocyte entry into the CNS. The dichotomy between clinical and pathological signs in the second active EAE experiment, with more severe symptomatology, may reflect higher levels of disease-promoting cytokines, such as TNF-α, acting directly on inflammatory events. There is documented evidence that the processing of TNF is blocked by MMP inhibitors [22, 23]. Variations in clinical scores between vehicle-treated groups do occur not infrequently and may be attributed to the animal batches themselves. It has recently been reported that Lewis rats vary considerably with respect to susceptibility to EAE, depending on the commercial source [24]. Furthermore, responses to the stress of sham treatment, which can activate the adrenal cortex, may also modulate the disease process [25]. The lack of an apparent effect of the MMP inhibitor on the pathology in the transfer model, despite a clinical benefit, may be the result of modulation at the level of effector but not of chemotactic cytokines produced in the CNS following transfer of lymphocytes from sensitized non-drug treated animals.

As the two models of EAE examined in the present study were acute in which the only histological feature is inflammation it was not possible to determine the effect of Ro31–9790 on demyelination. Histopathological studies by Gijbels et al [21] showed only a moderate decrease in demyelination in treated animals, suggesting that the inhibitor is unlikely to be acting at this level.

The present study confirms a role for MMPs in inflammation in EAE; however, at this point it is not possible to pinpoint single MMPs in the process since Ro31-9790 inhibits both gelatinase B and collagenase. Although demyelination is only moderate in EAE, long term studies of MMP inhibitors in chronic models may suggest whether there is any therapeutic potential for the human demyelinating disease, multiple sclerosis.

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