T-cell apoptosis in situ in experimental autoimmune encephalomyelitis following methylprednisolone pulse therapy

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Summary
The apoptosis-inducing effects of i.v. methylprednisolone were investigated as a possible method of controlling inflammation in the CNS in adoptive transfer-experimental autoimmune encephalomyelitis (AT-EAE) in Lewis rats. Two pulses of methylprednisolone were given at the peak of mild and of severe disease. T-cell apoptosis was assessed on spinal cord cross-sections by morphology and TUNEL staining. Concentrations of methylprednisolone were measured in serum, CSF and spinal cord tissue by high-pressure liquid chromatography (HPLC). In severe EAE, 10 mg/kg methylprednisolone increased T-cell apoptosis significantly and T-cell infiltration was marginally decreased. A maximal dose of 50 mg/kg methylprednisolone was superior in both respects and, in contrast to 10 mg/kg methylprednisolone, was also effective in mild EAE. A dose of 1 mg/kg methylprednisolone did not produce notable changes compared with controls treated with phosphate-buffered saline. Serum, CSF and spinal cord concentrations of methylprednisolone measured by HPLC 2 h after a single i.v. injection of 10 or 50 mg/kg methylprednisolone revealed significantly higher methylprednisolone concentrations in severe EAE compared with mild disease. With 50 mg/kg methylprednisolone, we obtained serum and CSF concentrations in the region of 10⁻⁵ M methylprednisolone. We also studied the expression of bcl-2, a typical anti-apoptotic regulatory protein, in T cells, and found no change after methylprednisolone treatment compared with controls. Methylprednisolone did not induce apoptosis of oligodendrocytes, which would have been an unwanted side effect in CNS cells. This study provides evidence that methylprednisolone dose-dependently augments T-cell apoptosis in situ in AT-EAE. Our results may have implications for the use of glucocorticosteroids at very high doses in the treatment of inflammatory disorders of the CNS, such as multiple sclerosis, or of other target organs.

Keywords: glucocorticosteroids; autoimmune disorders; inflammation; central nervous system; apoptosis

Abbreviations: AT-EAE = adoptive transfer-experimental autoimmune encephalomyelitis; BSA = bovine serum albumin; CNPase = 2',3'-cyclic nucleotide phosphohydrolase; GS = glucocorticosteroids; GSR = glucocorticoid receptor; HPLC = high performance liquid chromatography; MP = methylprednisolone; PBS = phosphate-buffered saline; TBS = Tris-buffered saline

Introduction
Glucocorticosteroids (GS) are potent anti-inflammatory drugs, and are commonly used in the treatment of autoimmune disorders. Their therapeutic efficacy has been established in immune neuropathies (Dyck et al., 1982; Pollard, 1987), multiple sclerosis (Durelli et al., 1986; Milligan et al., 1987; Myers, 1992), optic neuritis (Beck et al., 1992), lupus erythematosus (Mills, 1994) and cerebral vasculitis (Gross, 1994).
The pharmacological effects of GS are based on several mechanisms, leading to the modulation of cell activation, cytokine expression, the secretion of inflammatory mediators, leucocyte migration and the reduction of local oedema (Goldstein et al., 1992; Brann et al., 1995; Wehling, 1995). Furthermore, GS induce apoptotic cell death in susceptible cell types. Wyllie was the first to describe this effect for cultured thymocytes (Wyllie, 1980). T-cell apoptosis in situ after i.v. GS treatment was shown recently in experimental autoimmune neuritis (Zettl et al., 1994).

At low concentrations of GS, T-cell apoptosis is mediated by the glucocorticosteroid receptor (GSR) (Compton and Cidlowski, 1987; Schwartzman and Cidlowski, 1991; Osborne et al., 1996) and can be blocked by the GSR antagonist RU 468 (Grul and Altschmied, 1993; Gold et al., 1996). At higher doses, GS can exert proapoptotic effects by other mechanisms, such as binding to membrane receptors with the consecutive activation of a second messenger system, and by affecting the physicochemical properties of the cell membrane (Brann et al., 1995; Buttgereit et al., 1998). GS-mediated apoptosis was shown to be blocked by the antiapoptotic protein bcl-2 in vitro in thymocytes (Ivanov and Nikolic-Zugic, 1998) and partially blocked in human leukaemia cells (Hartmann et al., 1999).

Several clinical trials have investigated high-dose GS treatment in inflammatory diseases of the nervous system. Milligan demonstrated a clinical benefit from a 5-day course of i.v. methylprednisolone (MP) treatment in multiple sclerosis patients (Milligan et al., 1987). In acute optic neuritis, a 3-day course of 1000 mg i.v. MP followed by oral MP at 1 mg/kg per day for 11 days turned out to be slightly superior to 100 mg oral prednisone alone for 2 weeks (Beck et al., 1992). There is now evidence that high-dose GS therapy with 2000 mg MP i.v. is superior to 500 mg with regard to reduction of disease activity, as measured by MRI criteria (Oliveri et al., 1998).

Oestrogens, another group of steroid hormones, have attracted interest as possible therapeutic agents since higher concentrations of these hormones during pregnancy have been linked to lessened disease activity in female multiple sclerosis patients, and possibly a milder course of the disease (Damek and Shuster, 1997). In experimental autoimmune encephalomyelitis (EAE), 17β-oestradiol therapy delayed the onset of the disease (Jansson et al., 1994).

Here we formally investigated whether, in terms of induction of T-cell apoptosis in situ, maximal doses of MP are superior to the high doses previously used in the rat adoptive transfer-experimental autoimmune encephalomyelitis (AT-EAE) model and also in human therapy.

### Methods

#### Animals

For all experiments, female Lewis rats, aged 6–8 weeks and with a body weight of 140–160 g, were obtained from Charles River, Sulzfeld, Germany. Animals were housed in plastic cages without grid floors and were given commercial food pellets and water ad libitum.

All experiments were carried out in accordance with Bavarian state regulations for animal experimentation and were approved by the responsible authorities.

#### Cell culture

All culture media and supplements were obtained from Gibco BRL (Eggenstein, Germany). In vitro studies were performed on rat thymocytes. Apoptosis was studied in vitro following a previously reported method (Gold et al., 1994). In brief, freshly prepared thymocyte suspensions (5 x 10⁶ cells) were subjected to different concentrations of MP, fosfestrol or β-oestradiol, incubated at 37°C overnight and processed as described (Gold et al., 1994).

Encephalitogenic T cells for in vivo experiments were generated and maintained as described in detail previously (Gold et al., 1995). Primed T cells (3 x 10⁹/ml) were re-stimulated with guinea-pig myelin basic protein (20 µg/ml) in RPMI (Rosewell Park Memorial Institute medium) 1640 supplemented with 1% normal rat serum, 100 U/ml penicillin, 100 µg/ml streptomycin and 2 mM glutamine, using freshly isolated and irradiated (3000 rad) thymocytes (1.5 x 10⁶/ml) as the antigen-presenting cells. Seventy-two hours later, activated T-cell blasts were separated from cell debris by centrifugation on Ficoll gradients (Nycomed AS, Oslo, Norway).

#### Induction of EAE and clinical score

AT-EAE was induced by injection of freshly activated, myelin basic protein-specific T cells into the tail vein, using a dose of 2–4 x 10⁶ T cells to induce mild EAE and 8–12 x 10⁶ cells to induce severe EAE. Animals were weighed and inspected daily, using a six-grade score for clinical staging (0 = healthy; 1 = weight loss, limp tip of tail; 2 = limp tail, mild paresis; 3 = moderate paraparesis, ataxia; 4 = tetraparesis; 5 moribund; 6 = dead) (Lassmann, 1983).

Disease onset with first clinical signs was on day 2 (severe) or 3 (mild); a maximum of grade 2–3 was reached in mild EAE and grade 4–5 in severe EAE.

#### Treatment protocol and tissue sampling for apoptosis studies

The drugs used for in vivo and in vitro studies were methylprednisone-21-hydragensuccinate (MP) [Urbason soluble®; Hoechst (now Aventis Pharma), Frankfurt, Germany], fosfestrol (Honvan®; Asta Medica, Frankfurt, Germany) as a synthetic oestrogen and, for in vitro experiments only, β-oestradiol (Sigma, Deisenhofen, Germany).

The time schedule essentially followed a protocol that had
been established in earlier studies (Zettl et al., 1995). All experiments [except high-performance liquid chromatography (HPLC) analysis] were performed in groups of six animals each. At the peak of the disease, on day 4 or 5, the animals were injected i.v. with two pulses of MP at 1, 10 or 50 mg/kg body weight (controls) or with phosphate-buffered saline (PBS), 18 and 6 h before they were killed. To investigate late apoptotic effects in oligodendrocytes, we performed one experiment with two injections of 10 mg/kg MP at an interval of 12 h and perfused the animals 48 h after the first injection. In one experiment, fosfostrol at a dose of 300 mg/kg was compared with PBS.

Animals were anaesthetized with pentobarbital at 200 mg/kg body weight (Narcoren; Rhone Merieux, Laupheim, Germany) and perfused through the left cardiac ventricle with HAES-steril® 6% (Fresenius, Bad Homburg, Germany) for 5 min, followed by fixation with paraformaldehyde 4% in 0.1 M phosphate buffer at pH 7.4. The spinal cord was removed, postfixed for 1 h in the same fixative, cut into 5 mm slices, dehydrated and embedded in paraffin.

Immunohistochemistry: T cells, oligodendrocytes and albumin staining

Five micrometre cross-sections were deparaffinized with xylene and rehydrated. Hydroxylamine pretreatment (0.9% hydroxylamine in 0.05 M PBS) for 30 min was required only for the binding of the antibody in staining for albumin. Non-specific binding was prevented by incubation with 10% bovine serum albumin for 30 min. Immunohistochemistry was performed using a monoclonal mouse antibody to a pan-T-cell antigen (B 115–1; Holland Biotechnology, Leiden, Netherlands) diluted 1 : 500 in 0.05 M Tris-buffered saline (0.15 M sodium) (TBS) with 1% BSA (bovine serum albumin) for 1 h at room temperature. To detect disruption of the blood–brain barrier, we used an anti-albumin antibody (Nordic, Bochum, Germany) diluted 1 : 200 in 0.05 M PBS (0.15 M sodium), as described (Morrissey et al., 1996). Oligodendrocytes were stained with the mouse monoclonal antibody anti-CNPase (2’,3’-cyclic nucleotide phosphohydrolase) (Chemicon International, Hofheim, Germany), diluted 1 : 750 in TBS with 1% BSA for 2 h at room temperature. After blocking endogenous peroxidase activity with 3% H2O2 and 0.2 M sodium azide in methanol, primary antibodies were detected using the ABC system (DAKO, Hamburg, Germany) with 3,3’-diaminobenzidine tetrahydrochloride as substrate. All sections were counterstained with haematoxylin for 30 s, dehydrated and mounted in Vitro-clud® (R. Langenbrinck, Emmendingen, Germany).

Double-labelling techniques for detection of apoptosis and of bcl-2-positive T cells

Apoptotic T cells were identified by in situ tailing as described (Gold et al., 1994).

Labelling of cells in vitro was conducted by incubation in small reaction tubes containing 50 µl of 5 × tailing buffer (Promega, Heidelberg, Germany), 0.15 nmol fluorescein-12-dUTP (Boehringer, Mannheim, Germany), 0.85 nmol dTTP and 1 nmol dATP, dCTP and dGTP (Sigma). Five units of terminal transferase and water to 50 µl were added. Incubation of light-protected samples was performed at 37°C in a moist chamber on a rotating shaker for 1 h. We analysed 6000 gated events from the predominant small thymocyte population on a FACScan (Becton Dickinson, La Jolla, Calif., USA).

Tissue sections were deparaffinized, rehydrated and treated with chloroform for 1 s. To label apoptotic cells in the tissue, we used the Boehringer detection kit, following the instructions given by the supplier. For T-cell immunohistochemistry we followed the protocol given above, except that alkaline phosphatase-based detection was used with New-fuchsin (DAKO) as the chromogenic substrate. Sections were mounted in an aqueous mounting medium (Aquatex; Merck, Darmstadt, Germany).

Double staining for bcl-2 and T cells was performed on 5 µm paraffin sections, which were dewaxed and rehydrated following the protocol above. Preincubation in the microwave oven (850 W) was performed by boiling in 10 mM sodium citrate buffer, pH 6.0, for 30 min. After they had been cooled to room temperature, sections were washed thoroughly with distilled water followed by TBS. After blocking of non-specific binding with 10% BSA, sections were incubated with a polyclonal rabbit anti-bcl-2 antibody (Pharmingen, Hamburg, Germany) at a dilution of 1 : 750 in TBS with 1% BSA overnight at room temperature. As the secondary antibody, we used a biotinylated goat anti-rabbit IgG antibody (Vector, via Linaris, Wertheim, Germany), diluted 1 : 50 in TBS with 1% BSA. Visualization was achieved with an alkaline phosphatase AB system (DAKO) and Vector Red (Vector) as substrate. After the AB-binding sites had been blocked with an AB-blocking kit (Vector), T cells were detected with a biotinylated horse anti-mouse IgG antibody, which was preabsorbed with sera from rat, rabbit and goat for 15 min at 37°C and then diluted 1 : 100 in TBS with 1% BSA. The peroxidase-based AB system (DAKO) was used with diaminobenzidine–nickel (Vector) as the chromogenic substrate.

Analysis of inflammatory infiltrates and of apoptosis was performed by an observer blinded to the treatment, who rated 10 visual fields (1.6 mm²) or half a section of lumbar spinal cord at × 250 magnification, one section per animal. Apoptosis was assessed by morphological criteria (Wyllie, 1980) or labelling by in situ tailing. Apoptotic oligodendrocytes were determined in 0.01 mm² (10 visual fields at × 1000 magnification) of lumbar spinal cord by a blinded observer.

Semiquantitative analysis of disruption of the blood–brain barrier was performed as described recently (Morrissey et al., 1996), using a five-grade scale (0 = no staining; 1/2 = thin/thick ring of signal around the endothelial layer; 3 =
Table 1 Apoptosis of thymocytes in vitro

<table>
<thead>
<tr>
<th>Treatment</th>
<th>PBS</th>
<th>MP</th>
<th>Oestradiol</th>
<th>Fosfestrol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>27.1</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>1 µg/ml</td>
<td>–</td>
<td>97.8</td>
<td>–</td>
<td>27.2</td>
</tr>
<tr>
<td>3 µg/ml</td>
<td>–</td>
<td>–</td>
<td>35.9</td>
<td>49.3</td>
</tr>
<tr>
<td>10 µg/ml</td>
<td>–</td>
<td>97.3</td>
<td>32.2</td>
<td>40.2</td>
</tr>
<tr>
<td>30 µg/ml</td>
<td>–</td>
<td>–</td>
<td>35.9</td>
<td>49.3</td>
</tr>
<tr>
<td>100 µg/ml</td>
<td>–</td>
<td>–</td>
<td>47.7</td>
<td>68.4</td>
</tr>
<tr>
<td>300 µg/ml</td>
<td>–</td>
<td>–</td>
<td>71.4</td>
<td>80.7</td>
</tr>
</tbody>
</table>

Values for thymocyte apoptosis in relation to total thymocyte count after incubation with the concentrations indicated were obtained by FACS (fluorescence-activated cell sorter) analysis of TUNEL-positive thymocytes. Note the much higher concentrations needed with oestradiol and fosfestrol.

diffuse halo around vessels; 4 = more or less homogeneous staining signal).

Serum, CSF and tissue sampling for analysis of MP concentration by HPLC

For the analysis of MP concentration in serum, CSF and spinal cord, groups of three or four rats were injected once, 2 h before lethal anaesthesia with CO₂. First, blood was drawn by cardiac puncture. Then CSF was collected by puncture with a 1 ml syringe and 0.4 mm needle after preparation of the atlanto-occipital membrane. With few exceptions, samples were clear and not contaminated with blood. The spinal cord was removed for tissue analysis. All preparations of the atlanto-occipital membrane. With few exceptions, samples were clear and not contaminated with blood. The spinal cord was removed for tissue analysis. All samples were centrifuged after 30 min at room temperature, and serum was stored at –80°C together with all other probes. Concentrations of 6-α-methylprednisolone were measured by HPLC by Dr Jürgen Knöller, FOCUS Clinical Drug Development GmbH, Neuss, Germany, using coded samples. The method involved liquid/liquid extraction followed by HPLC with mass spectrometry/mass spectrometry detection, and was similar to that described in a previous report (Möllmann et al., 1995). Two calibration ranges were used: 0.1–10 ng/ml with a lower limit of quantification of 0.1 ng/ml for the CSF samples, and 10–1000 ng/ml with a lower limit of quantification of 10 ng/ml for the serum and spinal cord samples. In some animals, HPLC analysis of CSF was not possible because of the small amount of CSF, as indicated in the Results section.

Results

Steroid-induced apoptosis in vitro

In vitro experiments showed a rate of 97.8% apoptotic thymocytes after overnight incubation with 1 µg/ml MP versus 27.1% spontaneous apoptosis in untreated controls. In the presence of β-oestradiol or fosfestrol as source of oestrogen, apoptosis was seen only at much higher concentrations, and to a lesser degree (Table 1).

Steroid-induced apoptosis in EAE

For in vivo studies, cells were taken from the same restimulation cycle and the cell concentration was adjusted beforehand to standardize the severity of disease. Albumin staining correlated well with the clinical stage of EAE, which was rated as grade 1–3 in mild EAE and up to grade 3–4 in severe EAE (data not shown).

T-cell apoptosis after 10 or 1 mg/kg MP i.v.

In a first experiment, we compared MP at 1 and 10 mg/kg body weight and PBS in the treatment of severe EAE (Fig. 1). The 1 mg/kg MP group did not exhibit any significant change compared with the control. The group receiving 10 mg/kg MP showed a significant increase in apoptosis (P < 0.01 versus control), but only a trend towards reduced cellular infiltration.

In mild EAE, therapy with 1 or 10 mg/kg MP had no clear effect on T-cell apoptosis (mean ± SD: 1 mg/kg, 12.7 ± 5.9%; 10 mg/kg, 13.7 ± 5.3%; control, 9.3 ± 2.3%). T-cell infiltration was slightly reduced by 1 mg/kg MP (166.3 ± 86.0 versus 201.9 ± 125.4 cells/mm² for the control) and to a greater extent by 10 mg/kg MP (136.0 ± 47.2 cells/mm²), but the effect did not reach statistical significance. This experiment in mild EAE was repeated with similar results (data not shown).

T-cell apoptosis after 50 or 10 mg/kg MP i.v.

The following experiments were done to study very high doses, i.e. 10 and 50 mg/kg MP versus PBS (Fig. 2, cf. Fig. 5A–C). In severe EAE, 10 mg/kg MP significantly increased the rate of T-cell apoptosis (versus control, P < 0.05) (Fig. 5D and E), but 50 mg/kg had a greater effect (versus 10 mg/kg, P < 0.01). This was the first experiment in which we also observed a statistically significant decrease in T-cell infiltration after two injections of 50 mg/kg MP (versus control, P < 0.01), while 10 mg/kg again showed only a trend towards reduced infiltration. These results were reproduced in another set of experiments in animals with severe EAE (data not shown).

Next, we studied high-dose therapy in mild EAE (Fig. 3). At 10 mg/kg MP a slight augmentation of T-cell apoptosis was observed, and greater augmentation was found with 50 mg/kg (50 mg/kg MP versus control, P < 0.05). The
Steroid-induced T-cell apoptosis in EAE

Fig. 1 Treatment of severe EAE with 10 or 1 mg/kg MP or with PBS. (A) Clinical score. (B) Percentage of apoptotic T cells as rated by morphological criteria and TUNEL staining. (C) Number of infiltrating T cells per mm²; each symbol represents one animal and bars indicate mean ± SD. Apoptosis: **P < 0.01 (10 versus 1 mg/kg MP).

reduction in T-cell infiltration did not reach statistical significance. Similar results were obtained when the experiment was repeated (data not shown).

T-cell apoptosis after i.v. oestrogen treatment in severe EAE

The efficacy of oestrogens was studied in severe EAE. Fosfestrol at 300 mg/kg i.v. had no effect on T-cell apoptosis (12.7 ± 1.2% versus 11.2 ± 3.1% in the PBS group) or T-cell infiltration (145.4 ± 35.3 versus 133.6 ± 42.0 cells/mm² in controls). Since this treatment was not well tolerated and animals exhibited signs of cardiorespiratory dysregulation, we performed no further experiments with higher doses of fosfestrol or with other oestrogens (data not shown).

Measurement of MP concentrations in serum, CSF and spinal cord tissue

In order to show that the effects on T-cell apoptosis were indeed related to tissue concentrations of MP, we measured ex vivo concentrations in different compartments. Two experiments compared the effects of 10 versus 50 mg/kg MP in severe and mild EAE. For these studies, the rats were injected only once and sampling was performed 2 h later to achieve the presumed peak concentration (Kaiser and Kley, 1997). In both experiments, controls received i.v. injections of PBS, and in these animals 6-α-methylprednisolone was not detectable.

In the first experiment we compared three groups: 10 mg/kg MP in mild EAE, 10 mg/kg MP in severe EAE and 50 mg/kg MP in severe EAE. In severe EAE, 10 mg/kg MP resulted in much higher MP concentrations than 10 mg/kg MP in mild EAE (Fig. 4A), and 50 mg/kg MP in
severe EAE resulted in ~10-fold higher concentrations than 10 mg/kg MP in severe EAE (Fig. 4A).

In the second experiment, we treated mild EAE with 10 or 50 mg/kg MP. Again we found 10-fold higher MP concentrations after 50 than after 10 mg/kg MP treatment (Fig. 4B). In accordance with the previous experiment, the higher concentration of MP (50 mg/kg) gave a disproportionate, 10-fold increase in the serum concentration, which is explained by the typical non-linear pharmacokinetics of GS (Begg et al., 1987).

Expression of bcl-2 after steroid treatment
Double labelling for bcl-2 expression and T-cell antigens revealed no significant difference in the ratio of bcl-2-positive T cells 18 h after two injections of 10 mg/kg versus 1, 2 or 4 h after a single injection of 10 mg/kg. The rates of bcl-2 positive T-cell infiltration in the spinal cord or spleen were similar for the different treatments (Table 2 and Fig. 5F).

Oligodendrocyte apoptosis after steroid treatment
To investigate the effects of MP on other cells in the CNS that are susceptible to apoptosis, we studied oligodendrocytes. The rate of apoptosis was assessed on the basis of morphological criteria using CNPase immunohistochemistry. There was no increase in oligodendrocyte apoptosis 18 or 48 h after two i.v. pulses of 10 or 50 mg/kg MP (Table 3, Fig. 5G).

Discussion
Here we have demonstrated that high-dose i.v. GS treatment augmented T-cell apoptosis in situ in EAE. This effect was dependent on the dose of GS and the severity of the disease: in mild EAE, with less inflammation and less disruption of the blood–brain barrier, a standard high dose of MP, i.e. 10 mg/kg i.v., was in most cases not sufficient to increase T-cell apoptosis clearly, whereas a 5-fold higher dose of MP was consistently effective. In contrast, when rats suffered from severe EAE, both 10 and 50 mg/kg augmented T-cell apoptosis in situ, but 50 mg/kg MP had the greater effect. At 1 mg/kg, MP did not affect T-cell apoptosis in any of the experimental settings. Also, the synthetic oestrogen fosfestrol did not increase the rate of apoptosis.

Probably as a consequence of increased T-cell apoptosis, inflammatory T-cell infiltration was markedly reduced. A study of the regulatory protein bcl-2 did not show a notable change in the number of bcl-2-positive T cells shortly after injection. There were no unwanted side-effects on glial cells such as oligodendrocytes. We might have seen effects of the decrease in inflammation on the clinical course if we had followed the animals for a longer period into recovery, but this was not possible because of the experimental design. In a recent study, a dose-dependent effect of GS-induced apoptosis in active EAE was also demonstrated (Nguyen et al., 1997). No beneficial effect on the severity of the relapse was observed.

A possible pharmacological mechanism to explain our findings is the higher level of MP that we found in the CSF and spinal cord, which could lead to qualitatively different effects of GS. Concentrations of MP in serum and spinal cord tissue, as measured by HPLC, correlated well with the induction of T-cell apoptosis. With 50 mg/kg MP, mean humoral and tissue concentrations of up to $3.3 \times 10^{-5}$ M were achieved, which is in the range of high-dose effects according to a dose-dependent model of steroid action (Buttgereit et al., 1998). At 10 mg/kg, concentrations in the range of $5 \times 10^{-7}$ to $10^{-6}$ M were obtained. The non-linear
increase in MP concentration after 50 mg/kg MP treatment compared with 10 mg/kg is explained by the typical pharmacokinetics of GS, which exhibit dose-dependent distribution volumes, half-life in plasma and metabolic clearance (Begg et al., 1987; Kaiser and Kley, 1997). Treatment with 10 mg/kg MP led to higher serum concentrations in severe EAE than in mild EAE, which may have been due to the dehydration of the severely diseased animals.

The effects of MP on T-cell apoptosis could have been due to interaction with apoptosis regulatory proteins (Ivanov and Nikolic-Zugic, 1998). Investigation of bcl-2-expressing T cells by immunohistochemical double labelling revealed no change for the antiapoptotic bcl-2 at any time after...
administration of i.v. 10 mg/kg MP compared with untreated controls. These results are in accordance with a recent observation in which hydrocortisone-induced apoptosis in the murine thymus was not associated with altered bcl-2 expression (Denis et al., 1998). This may indicate the existence of factors regulating GS-induced T-cell apoptosis, such as the direct activation of caspases.

To test the possibility that MP would increase the apoptosis not only of T cells but also of glial cells, we analysed oligodendrocytes immunohistochemically. We noted that MP
did not induce apoptosis of oligodendrocytes as an unwanted side-effect, even when we looked at late time points after MP administration.

GS have been shown to exert their multiple actions via different pathways. In general, these mechanisms act at the genomic or non-genomic level. The classical, genomic actions occur at low doses, are GSR-mediated and can be blocked by the GSR antagonist RU 468 (Gruol and Altschmied, 1993; Gold et al., 1996). To date, it is unclear whether GS-mediated T-cell apoptosis can also be exerted by non-genomic actions. Non-genomic mechanisms of GS, such as the direct alteration of membrane fluidity, binding to specific cell membrane receptors and the use of cyclic adenosine monophosphate as a second messenger (Rosner et al., 1999), and other pathways (Brann et al., 1995), operate at very high doses (Buttgereit et al., 1996), which were achieved in this study. Another possible mediator of GS-induced apoptosis is calcium.

### Table 2 Percentage of bcl-2-positive T cells after MP treatment in vivo

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>1 h</th>
<th>2 h</th>
<th>4 h</th>
<th>18 + 6 h</th>
</tr>
</thead>
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<tr>
<td>Spinal cord</td>
<td>16.5 ± 4.3</td>
<td>11.2 ± 5.5</td>
<td>13.0 ± 9.1</td>
<td>16.2 ± 0.4</td>
<td>13.9 ± 6.9</td>
</tr>
<tr>
<td>Spleen</td>
<td>22.6 ± 3.1</td>
<td>17.0 ± 6.3</td>
<td>16.7 ± 3.3</td>
<td>19.9 ± 5.9</td>
<td>17.5 ± 8.4</td>
</tr>
</tbody>
</table>

Immunohistochemical analysis by double labelling for bcl-2 and T-cell markers. Data (mean ± SD) are percentages of bcl-2-positive T cells in the spinal cord and spleen at the times indicated after a single injection of 10 mg/kg MP and after two injections of 10 mg/kg MP in groups of three rats.

### Table 3 Percentage of apoptotic oligodendrocytes after MP in vivo

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>MP 10 mg/kg</th>
<th>MP 50 mg/kg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mild EAE, 18 h</td>
<td>12.0 ± 5.1</td>
<td>14.9 ± 5.3</td>
<td>9.2 ± 1.5</td>
</tr>
<tr>
<td>Severe EAE, 18 h</td>
<td>14.3 ± 5.3</td>
<td>11.4 ± 3.7</td>
<td>14.9 ± 5.2</td>
</tr>
<tr>
<td>Severe EAE, 48 h</td>
<td>7.7 ± 2.8</td>
<td>–</td>
<td>8.8 ± 2.0</td>
</tr>
</tbody>
</table>

Immunohistochemical analysis of oligodendrocytes. Data (mean ± SD) are percentages of morphologically apoptotic oligodendrocytes in the spinal cord after two injections of 10 or 50 mg/kg MP at 18 and 6 h or at 48 and 36 h before perfusion in groups of six rats.

Recently, two cationic membrane channels, the ATP-gated P2X1 receptor (Chvatchko et al., 1996) and the inositol 1,4,5-trisphosphate (IP3) receptor (Khan et al., 1996), have been shown to be upregulated in GS-induced apoptosis of thymocytes. At present it is unclear whether this mechanism also contributes to the effects of very high doses of MP.

It remains unclear whether non-genomic actions of GS can contribute to their anti-inflammatory effects in the therapy of autoimmune diseases (Buttgereit et al., 1998). In humans, GSR are saturated at a dose of 2.5–4 mg/kg prednisone equivalent (Buttgereit et al., 1996). Because of the different surface areas of humans and rats, in rats some drugs might need a dose up to 7-fold higher compared with humans (Klaassen et al., 1986). This depends on the drug as well as on the pharmacological effect investigated. For corticosteroids, no exact data are available, but the saturation of all GSR in rats should be reached with 10 mg/kg MP, accounting for the non-genomic effects observed with very high doses in this study. In this respect, the dose-dependent induction of apoptosis shown here can help to understand the superior efficacy of very high dose MP therapy in multiple sclerosis. A similar large effect of high-dose MP pulse therapy on the blood–brain barrier, as reflected by the decrease in enhancing lesions seen on MRI, has been shown previously (Oliveri et al., 1998). Except for apoptosis and the integrity of the blood–brain barrier, other mechanisms, such as the downregulation of metalloproteinases and the modulation of the cytokine profile, may account for the dose-dependent effects of MP seen here in EAE. The peak of the AT-EAE disease studied here is typically accompanied by upregulation of strong proapoptotic factors, such as TNF-α (tumour necrosis factor-α), which is obviously implicated in T-cell apoptosis in the CNS (Bachmann et al., 1999). Therefore, we postulate an overall additive effect of MP on T-cell apoptosis which may act in concert with the counteraction of activation-induced T-cell apoptosis by steroids, as indicated by McCombe and colleagues (McCombe et al., 1996).

However, little is known about the dose-dependent modulation of different mechanisms by very high (50 mg/kg) and high (10 mg/kg) doses of MP. Also, the clinical relevance of the different mechanisms and their modulation by MP in relapses of multiple sclerosis remains unclear and needs to be investigated.
be addressed in a chronic–relapsing model of EAE. In AT-EAE we did not observe relapses when rats were followed for a prolonged period after pulse therapy.

Our results may have implications for the therapy of acute relapses of multiple sclerosis and possibly of other autoimmune disorders. In some cases the standard regimen for the treatment of relapses with 10 mg/kg MP i.v. might not be sufficient to eliminate autoaggressive T cells in the lesion, especially in milder relapses with little disruption of the blood–brain barrier and inflammation. Very high doses may help to terminate inflammation more rapidly and might prevent ongoing immune reactions such as epitope spreading (Lehmann et al., 1993). This may also help to explain the surprising observation made in the optic neuritis trial, in which a reduced rate of development of multiple sclerosis was documented over a 2-year follow-up period in a group receiving 10 mg/kg MP (Beck et al., 1993). Equivalent very high doses have already been used in the therapy of acute spinal cord injury. In this setting, 100 mg as well as 1000 mg MP failed to show beneficial effects, whereas a 30 mg/kg bolus followed by 5.4 mg/kg per h for 23 h improved motor function and sensation significantly (Bracken et al., 1990, 1997). No serious adverse reactions of this regimen were noted on retrospective analysis (Gerndt et al., 1997).

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