High-Dose Methylprednisolone Therapy in Multiple Sclerosis Induces Apoptosis in Peripheral Blood Leukocytes

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Background: Apoptosis is supposed to contribute to the elimination of T cells from the inflamed central nervous system in the natural disease course of multiple sclerosis (MS). In the animal model experimental autoimmune encephalomyelitis, T-cell apoptosis can be induced by high-dose glucocorticoid (GC) administration.

Objective: To study the effects of intravenous high-dose GC therapy in MS on T-cell apoptosis ex vivo.

Patients: Sixty-six patients with MS (28 with relapsing-remitting MS, 22 with secondary chronic progressive MS, and 16 with primary chronic progressive MS) and 16 control patients receiving corticosteroids for other disorders were included in the study.

Methods: Blood samples were collected before and immediately after the first infusion of 500 to 1000 mg of methylprednisolone given during 2 hours in the early morning. Gradient-isolated peripheral blood leukocytes (PBLs) were cultured, unstimulated, with corticosteroids (positive control), the mitogen phytohemagglutinin, or anti–T-cell receptor monoclonal antibody. For investigation of apoptosis, PBLs were cultured overnight and analyzed by immunoflow cytometry using TUNEL (terminal transferase-mediated dUTP biotin nick end labeling) or annexin labeling in combination with CD4, CD8, CD22, CD56, or bcl-2 staining. Proliferation was measured by ³H-thymidine incorporation. For cytokine determination, supernatants were collected after 48 hours of culture.

Results: After in vivo corticosteroid treatment, apoptosis of unstimulated PBLs was markedly and significantly augmented in all 3 MS subgroups. Fluorescence-activated cell sorter analysis showed that apoptosis affected predominantly CD4 T cells. Natural killer cells showed a relative increase after GC therapy without a change in the rate of apoptotic cells. Expression of bcl-2 in T-cell subpopulations was not significantly modified by high-dose GC therapy. Culture supernatants of T-cell receptor–stimulated PBLs after GC therapy contained lower concentrations of interleukin 2, interferon gamma, and tumor necrosis factor α than those from PBLs taken before pulse therapy. Similar changes in the rate of apoptosis and cytokine production were seen in controls.

Conclusions: Corticosteroid pulse therapy is a strong inducer of leukocyte apoptosis. Induction of apoptosis might contribute to the down-regulation of T-cell activity and thereby terminate inflammation in the central nervous system.

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THERE IS EVIDENCE that multiple sclerosis (MS) is a disease in which, among others, autoimmune processes play a central role. Inflammatory attack results from the interaction of T lymphocytes with other components of the immune system. Intravenous (IV) treatment with high-dose glucocorticoids (GCs) has now become the standard therapy in patients with an acute relapse of disease or a rapidly progressive deterioration of chronic progressive MS. Short-term high-dose corticosteroid therapy does not affect the hypothalamic-pituitary-adrenal axis in these patients and does not reduce bone density.

Several studies on GC treatment yielded controversial results as to the most effective dose and form of application. Results of the North American Optic Neuritis Treatment Trial indicated that high-dose IV methylprednisolone treatment slightly accelerates recovery from relapses and might even reduce the risk of subsequent attacks, whereas low-dose oral prednisolone therapy is ineffective and might even be associated with an increased risk of subsequent development of MS. In a small, double-blind, controlled trial, the effect of 500 mg of methylprednisolone administered orally for 5 days was not different from giving the same dosage intravenously.

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PATIENTS, MATERIALS, AND METHODS

PATIENTS

A total of 66 patients (49 women and 17 men) with MS were included in this study: 28 (21 women and 7 men) with relapsing-remitting (RR) MS, 22 (19 women and 3 men) with secondary progressive (SP) MS, and 16 (9 women and 7 men) with primary progressive (PP) MS. Sixteen patients (10 women and 6 men) who received GC pulse therapy for other disorders, such as inflammatory neuropathy,3 plexus neuritis,7 vasculitis,1 lumbar disc herniation,1 myositis,1 myelitis,1 and papillitis,1 served as control subjects.

The mean ± SEM ages of the groups were 35 ± 2 years for the RRMS patients, 44 ± 2 years for the SPMS patients, 31 ± 2 years for the PPMS patients, and 31 ± 4 years for the control group.

Of the 66 MS patients, 9 (5 with SPMS and 4 with RRMS) were pretreated with azathioprine (100-200 mg/d), 2 (both with SPMS) with cyclophosphamide, 2 (both with RRMS) with β-interferon, and 1 (with SPMS) with methotrexate (7.5 mg/wk) in a long-term immunosuppressive therapy regimen for more than 12 months, ie, in a steady state situation.

Except for one patient receiving 8 mg of methylprednisolone orally daily, the patients had not undergone GC pulse therapy for a minimum of 2 months.

METHODS

GC Pulse Therapy

Typically, 1000 mg of methylprednisolone (Urbason; Hoechst, Frankfurt, Germany) IV over 3 days (300 mg IV over 5 days in 6 MS patients and 6 controls and 250 mg IV over 5 days in 5 controls) was given in the morning with an infusion time of 2 hours. Blood samples (20 mL of EDTA) were taken by venipuncture from each patient before and immediately after the first GC infusion, taking into account the plasma half-time for methylprednisolone of 1.5 to 3 hours. The indication for GC therapy was made by physicians not involved in this study. The study was approved by the local ethics committee, and consistent informed consent was obtained.

Cell Culture

All culture media and supplements were purchased from Gibco BRL (Eggenstein, Germany). Immediately after blood samples had been taken, peripheral blood leukocytes (PBLs) were separated by gradient centrifugation using Ficoll (Nycomed AS, Oslo, Norway), washed twice, and seeded at 4 × 10^5 cells per well in 96-well round bottom microtiter plates (Nunc, Wiesbaden, Germany) in 100 µL of standardized cell culture medium (RPMI-1640 supplemented with glutamine, 2 mmol/L; penicillin G sodium, 100 U/mL; streptomycin sulfate, 100 µg/mL; and 5% fetal calf serum).

In unstimulated cultures, the percentage of apoptotic CD4 T cells increased from 2.3% ± 0.2% to 4.5% ± 0.4% in RRMS (P < .01), from 2.1% ± 0.2% to 3.5% ± 0.3% in SPMS (P < .01), and from 2.6% ± 0.4% to 3.6% ± 0.5% in PPMS (P < .05) as measured by TUNEL (terminal transferase-mediated dUTP biotin nick end labeling) (Figure 1). The 2 methods of TUNEL staining and annexin labeling yielded comparable results. When GCs were added directly to the culture, similar rates of apoptosis were observed from cells taken before and after pulse therapy (data not shown), speaking for a similar corticosteroid susceptibility of PBLs taken before pulse therapy.

RESULTS

METHYLPREDNISOLONE PULSE THERAPY IN VIVO AUGMENTS PBL APOPTOSIS IN VITRO

We found a significant increase of apoptotic PBLs in all subgroups of MS after IV high-dose GC treatment.
Pharmacia Biotech, Freiburg, Germany). Five units of terminal transferase and distilled water to 50 µL were added. After incubation for 1 hour at 37°C on a rotating shaker, immunofluorescence was measured with a fluorescence-activated cell sorter (FACScan; Becton Dickinson, Heidelberg) using the CellQuest software (Becton Dickinson).

For bcl-2 double staining, PBLs were fixed (see the previous paragraph) and then incubated with a hamster anti–human bcl-2 IgG antibody (Pharmingen, Hamburg, Germany) and fluorescein isothiocyanate–labeled goat anti–hamster IgG antibody (Dianova, Hamburg) at a dilution of 1:100, followed by addition of a mouse anti–human CD4 antibody (IgG1; Becton Dickinson, San Jose, Calif) detected with goat anti–mouse IgG1-Tricolor (Medac, Hamburg) or addition of mouse anti–human CD8 antibody (IgG2a; Becton Dickinson) labeled with goat anti–mouse IgG2a-Tricolor (Medac) to achieve differential detection on a FACSscan. The antibodies used for fluorescence-activated cell sorter analyses are listed in Table 1.

After overnight culture, apoptotic cells were detected by surface labeling with annexin–fluorescein isothiocyanate (Boehringer). A total of 2 × 10⁵ cells were incubated in tubes with 5 mmol/L Ca²⁺ buffer. After addition of annexin according to the instructions of the supplier in a final concentration of 2.5 µg/mL, incubation at 4°C for 10 minutes, and washing, cells were light protected and diluted in 80 µL of phosphate-buffered saline solution, 1% bovine serum albumin, and 0.1% sodiumazide for further processing. Peripheral blood leukocytes were then incubated with either anti–human CD4 Tricolor/anti–human CD22 phycoerythrin or anti–human CD8 Tricolor/anti–human CD36 phycoerythrin at 4°C for 10 minutes, washed, and measured by fluorescence-activated cell sorter analysis.

Proliferative responses were determined by ³H-thymidine uptake. After 48 hours, cultures were pulsed with ³H-thymidine, 0.007 MBq/well (Amersham-Buchler, Braun- schweig, Germany), for 16 hours. The cells were then collected on fiberglass filter paper in a Betaplate 96-well harvesting (Pharmacia Biotech), and the incorporated radioactivity was quantified in a Betaplate 96-well liquid scintillation counter (Pharmacia Biotech). Values were expressed as counts per minute (mean of triplicate cultures).

### Cytokine Analysis by Enzyme-Linked Immunosorbert Assay

For determination of cytokine production by cultured PBLs (interleukin [IL]-2, interferon gamma [IFN-γ], and tumor necrosis factor α [TNF-α] as T₃₁ cytokines and IL-4 and transforming growth factor β [TGF-β] as T₄₂ cytokines), supernatants of unstimulated and anti-CD3–stimulated cells were collected after 48 hours of culture and stored at –80°C.

Transforming growth factor β was measured in acid-activated supernatants in duplicates using a diagnostic enzyme-linked immunosorbert assay (ELISA) kit (Genzyme, Karlsruhe, Germany).

For measurement of IL-2 and IL-4, Duosets (Genzyme) were used; for measurement of IFN-γ and TNF-α, complementary antibody pairs and standards (R&D Systems, Abingdon, England) were adapted for optimal sensitivity of the ELISA following the instructions given by the suppliers. Concentrations of these cytokines in the supernatants were determined in triplicate, and probes of 1 donor (before and after GC infusion) were always measured in the same assay to exclude interassay variation.

### Statistical Analysis

Statistical analysis was performed using a software program (GraphPad Prism TM, Version 2.0; GraphPad Software Inc, San Diego, Calif). After checking for a symmetric distribution of data, the t test for grouped data was used, with P<.05 and P<.01 considered statistically significant. Data are given as mean±SEM. After classification of cytokine modulation by GC infusion (increase of >20%, decrease of >20%, and unchanged [increase or decrease ≤20%]), results were tested by the Dixon and Mood test for statistical significance.

### Table 1. Cell Specificity, Concentration and Dilution, and Supplier of Antibodies Used for Fluorescence-Activated Cell Sorter Analysis

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Cell Specificity</th>
<th>Concentration, Dilution</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti–human CD4 Tricolor</td>
<td>Helper T cell</td>
<td>1:10</td>
<td>Pharmingen, Hamburg, Germany</td>
</tr>
<tr>
<td>Anti–human CD8 Tricolor</td>
<td>Cytotoxic T cell</td>
<td>1:10</td>
<td>Pharmingen</td>
</tr>
<tr>
<td>Anti–human CD22 phycoerythrin</td>
<td>B cell</td>
<td>1 mg/mL, 1:10</td>
<td>Becton Dickinson, San Jose, Calif</td>
</tr>
<tr>
<td>Anti–human CD56 phycoerythrin</td>
<td>Natural killer cell</td>
<td>12.5 µg/mL, 1:10</td>
<td>Immunotech, Marseille, France</td>
</tr>
<tr>
<td>Anti–human CD3 (X5) IgG2a (mouse)</td>
<td>T cell</td>
<td>0.1 µg/mL, 1:10</td>
<td>Coulter, Krefeld, Germany</td>
</tr>
<tr>
<td>Anti–human bcl-2 IgG1 (mouse)</td>
<td>Human bcl-2</td>
<td>0.5 mg/mL, 1:100</td>
<td>Pharmingen</td>
</tr>
</tbody>
</table>

After GC therapy; P<.01, in SPMS (3.8%±0.8% vs 6.6%±1.2%; P<.01), and to a lesser degree in PPMs (4.3%±1.1% vs 6.2%±1.2%; P=.02). Concerning the number of CD4 T cells, IV GC therapy caused a marked and significant reduction of CD4 T cells in PBLs of all MS patients (after an in vitro culture even in the absence of GC therapy), which did not differ between the various MS subgroups (RRMS, 47.1%±2.8% before vs 27.9%±3.0% after GC therapy; SPMS, 54.4%±4.6% before vs 36.5%±4.9% after GC therapy; and PPMS, 49.9%±2.3% before vs 31.2%±4.4% after GC therapy; P<.001 for each group).

Because of the marked reduction of CD4 T cells, the proportion but not the absolute number of CD8...
T cells in MS patients tended to increase after GC infusion in RRMS (35.6%±2.2% before vs 39.0%±2.9% after GC therapy; P=.2) (Figure 2), (more clearly) in PPMS (31.7%±3.1% before vs 41.0%±4.1% after GC therapy; P=.03), and (significantly) in SPMS (32.9%±3.4% before vs 40.7%±3.6% after GC therapy; P<.01). Flow cytometric double labeling of apoptotic CD8 T cells also revealed a significant increase in RRMS (3.9%±0.3% before vs 5.7%±0.7% after GC therapy; P=.03) and in SPMS (4.5%±0.7% before vs 6.9%±0.9 after GC therapy; P=.01) but not in PPMS (6.3%±1.8% before vs 6.8%±1.3% after GC therapy; P=.64).

Natural killer (NK) cells exhibited a significant relative increase after GC pulse therapy (RRMS, 28.2%±3.4% before vs 47.5%±4.02% after GC therapy [Figure 2]; SPMS, 24.2%±3.9% before vs 40.1%±5.3% after GC therapy; and PPMS, 27.5%±4.2% before vs 48.4%±6.6% after GC therapy; P<.01 for all groups). However, the rate of apoptotic NK cells was not changed by precedent GC infusion. We did not find significant changes in the relative number of B-cell lymphocytes in any of the groups after methylprednisolone pulse therapy. The percentage of apoptotic B cells revealed a significant increase only in patients with SPMS (16.2%±11.1% before vs 22.7%±11.9% after methylprednisolone therapy; P=.03).

Because NK cells are partly positive for CD4, the total number of cells staining for CD4, CD8, NK, and B cells in some patients exceeded 100%.

EXPRESSION OF bcl-2 IN T-CELL SUBPOPULATIONS WAS NOT MODIFIED BY GC THERAPY

In any group of patients, analysis of bcl-2 expression in subpopulations of lymphocytes by double staining in combination with CD4 or CD8 did not show a significant effect of GC pulse therapy (data not shown). Note that these analyses were performed after 16 hours of cell culture only, and thus early changes in bcl-2 expression were not studied.

METHYL PREDNISOLONE PULSE THERAPY REDUCES CELLULAR PROLIFERATION

On stimulation with the mitogen phytohemagglutinin, PBLs isolated after GC pulse therapy exhibited significantly reduced cellular proliferation (RRMS, 14 708±1773 cpm before vs 5 118±1355 cpm after GC therapy; SPMS, 12 555±1621 cpm before vs 5 429±1695 cpm after GC therapy; P<.01 for both). In PPMS, the reduction of cellular proliferation after GC treatment was not significant (8 097±1470 cpm before vs 6 450±1589 cpm after GC therapy; P=.25). Stimulation with the T-cell receptor–activating monoclonal antibody X35 revealed comparable results in RRMS and SPMS (P<.01 but also displayed significant effects in PPMS (P=.02). An example of the proliferation data from X35-stimulated PBL cultures is illustrated in Figure 3. Similar results were seen in unstimulated PBL cultures and after addition of meth-
yprednisolone to cultures, yet overall incorporated radioactivity was less than 1000 cpm.

**HIGH-DOSE METHYLPREDNISOLONE INHIBITS THE PRODUCTION OF T_{H1} BUT NOT T_{H2} CYTOKINES BY T-CELL RECEPTOR–STIMULATED PBLs**

Analysis of cytokine secretion of T-cell receptor–stimulated PBLs from 41 MS patients from any subgroup showed a decrease of the T_{H1} cytokines IL-2, IFN-γ, and TNF-α after GC pulse therapy by more than 20% in most of the cultures, which all reached statistical significance (P<.05-.01). In contrast, the T_{H2} cytokines IL-4 and TGF-β were not remarkably affected by GC therapy (Table 2).

Cytokine levels in the various subgroups of MS before and after GC therapy in unstimulated and X35-stimulated cultures are illustrated in Figure 4.

**APOPTOSIS OF PBLs, DECREASE OF CD4 T CELLS, AND REDUCED PROLIFERATION AND T_{H1} CYTOKINE PRODUCTION RATE AFTER METHYLPREDNISOLONE PULSE THERAPY ARE NOT SPECIFIC FOR MS PATIENTS**

In controls with inflammatory and noninflammatory disorders, GC pulse therapy also led to a marked and significant increase of apoptotic PBLs (2.4%±0.4% before vs 3.2%±0.6% after GC therapy; P=.02) and to a significant decrease of CD4 T cells (52.5%±2.6% before vs 34.6%±3.0% after GC therapy; P<.01). The increase of NK cells after high-dose GC therapy (24.8%±3.0% before vs 46.3%±3.4% after GC therapy) and of CD8 T cells (31.9%±1.7% before vs 42.4%±2.3% after GC therapy; P<.01 for both) was comparable to the results in MS patients. Similar to MS patients, the rate of apoptotic CD8 T cells was not affected by GC therapy (P=.9), whereas the rate of apoptotic CD4 T cells increased from 4.8%±0.7% before to 8.3%±0.75% after GC therapy (P<.01).

Proliferation assays of unstimulated (737±168 cpm before vs 4057±76 cpm after GC therapy; P<.01), phytohemagglutinin-stimulated (9296±1863 cpm before vs 3764±1196 cpm after GC therapy; P<.01), and X35-stimulated (8589±2198 cpm before vs 3828±1011 cpm after GC therapy; P=.02) PBL cultures displayed a significant decrease of cellular proliferation after GC pulse therapy.

**COMMENT**

Glucocorticoids are potent anti-inflammatory drugs commonly used in the treatment of autoimmune disorders. In this study, we investigated the effects of high-dose IV GC therapy on subsequent PBL apoptosis in vitro and on leukocytic production of cytokines in vitro in MS patients and in non-MS patients receiving GC for other indications. Using flow cytometry and proliferation assays, we demonstrated that GC pulse therapy rapidly triggers PBLs in vivo for subsequent apoptosis in vitro. CD4 T cells showed the highest rate of apoptosis in vitro. Besides T-cell migration in vivo, the observed susceptibility of CD4 T cells to corticosteroid treatment and apoptosis of CD4 T cells might partly be the reason for the preferential decrease of circulating CD4 T cells after GC infusion.22-25

Previous investigators revealed a transient decrease of CD4 T cells and concomitant down-regulation of IL-2 and IFN-γ after high-dose IV methylprednisolone therapy in MS patients26,27 and a decrease of proliferation of CD45RA⁺ T cells after high-dose methylpred-
Glucocorticoid (GC) pulse therapy. A, Interleukin (IL)-2; B, IL-4; C, interferon gamma (IFN-γ); D, tumor necrosis factor α (TNF-α); and E, transforming growth factor β (TGF-β). Asterisks indicate a significant decrease. Data represent mean ± SEM.

Anti-inflammatory effects of GC therapy on immune cells may result in cell death or anergy. Both conditions are associated with reduced proliferative capacity of PBLs. This was shown by 3H-thymidine incorporation. This reduction most likely exceeded the extent that may be explained by apoptosis and favors other potent antiproliferative mechanisms of GC. To elucidate these mechanisms, we investigated the effects of corticosteroids on cytokine production using ELISA and revealed, in accord with other authors, inhibitory effects on the secretion of IL-2, IFN-γ, IFN-α, and TNF-α. The T1ε2 cytokines IL-4 and TGF-β were not significantly affected by GC. In a recent study with 18 MS patients, Wandinger et al focused on the production of IL-1, IL-2, IFN-γ, IFN-α, and TNF-α after GC pulse therapy and measured a decrease of all mentioned cytokines. The selective effects of methylprednisolone on the expression of T1ε1 cytokines may relate to different numbers of GC receptors or different GC receptor affinities in T1ε1 and T1ε2 cells. Besides this possibility, GC sensitivity may change in relation to the activation stage of the respective T-cell subsets.

The effects of GC pulse therapy on the rate of apoptotic CD4 and CD8 T cells and on the reduction of cellular proliferation were comparable in RRMS and SPMS patients but less obvious in PPMS patients. This might be an explanation for the limited efficacy of GC therapy on the course of the disease in the latter patients. In principle, concomitant immunosuppressive treatment could have impact on the results of our study. This reflects the daily situation in the clinic. Because patients were all being treated for more than 12 months, we assumed that they had achieved a stable condition.

Bcl-2 protein enhances cell survival in several experimental systems through the inhibition of apoptosis and might explain the differential susceptibility of T-cell subsets to corticosteroid-induced apoptosis. Expression of bcl-2 was not different between the CD4 and CD8 T cells, in contrast to the findings of Migita et al, who detected higher levels of bcl-2 in CD8 T cells. The fact that the percentage of bcl-2–expressing T cells in CD4 and CD8 T cells was not change after high-dose methylprednisolone therapy in our study suggests that increased apoptosis under GC infusion is not due to the downregulation of bcl-2 by GC. Recently, Schmitz et al presented evidence for 2 different cell types (CD95 type 1 and type II cells), of which only type II cells showed a reduction of apoptosis due to overexpression of bcl-2. This implies that bcl-2 might rescue cells from apoptosis only in certain PBL subpopulations. Alternatively, one has to consider that our bcl-2 analyses were not performed directly after GC therapy but in cells cultured for 16 hours. Thus, early changes might have been missed.

In MS patients, elimination of inflammatory T cells by apoptosis occurs as a physiologic defense mecha-


