Methylprednisolone Acts on Peripheral Blood Mononuclear Cells and Endothelium in Inhibiting Migration Phenomena in Patients With Multiple Sclerosis

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Background: Intravenous methylprednisolone hemisuccinate is administered to patients with multiple sclerosis (MS) during exacerbations to improve the rate of recovery. Corticosteroids could be beneficial in MS exacerbations also by decreasing transmigration of peripheral blood mononuclear cells (PBMNCs) through the blood-brain barrier.

Objectives: To evaluate how in vivo intravenous methylprednisolone treatment in patients with MS could influence transmigration of PBMNCs in an in vitro model; to perform transmigration experiments through a methylprednisolone-treated endothelium with PBMNCs from untreated healthy control subjects to evaluate putative selective effects of corticosteroids on endothelium; concomitantly, to quantify the concentration of matrix metalloproteinases 2 and 9 in supernatants of PBMNCs and in serum samples from methylprednisolone-treated patients with MS; to evaluate monokine induced by interferon-γ release in the supernatants of human umbilical vein endothelial cells treated with interferon-γ alone or interferon-γ and methylprednisolone; and to perform gene expression studies of matrix metalloproteinases 2 and 9 in human umbilical vein endothelial cells and PBMNCs from methylprednisolone-treated patients with MS.

Patients: Eight patients with MS in exacerbation were studied before and 3 and 24 hours after intravenous methylprednisolone treatment, 1 g.

Results: The absolute number of transmigrated PBMNCs from methylprednisolone-treated patients with MS significantly (P<.01) decreased at 3 hours and increased again at 24 hours, reaching values higher than those before treatment onset. Methylprednisolone was also able to significantly (P<.03) reduce the number of PBMNCs from healthy controls migrating through interferon-γ-stimulated or unstimulated endothelium. In vitro methylprednisolone treatment decreased monokine induced by interferon-γ production in human umbilical vein endothelial cells.

Conclusions: Methylprednisolone may be able to decrease transmigration of PBMNCs through the blood-brain barrier, exerting its inhibitory effects on PBMNCs and endothelium. A “rebound” of transmigration at 24 hours suggests that a single infusion is not optimal for achieving a persistent reduction in transmigration.

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PATIENTS, MATERIALS, AND METHODS

HUMAN UMBILICAL VEIN ENDOTHELIAL CELL ISOLATION

Human umbilical vein endothelial cells (HUVECs) were obtained from healthy donors (spontaneous childbirth). Cells were isolated according to the method of Jaffe et al. Culture purity was tested by von Willebrand factor antigen staining.

IN VIVO TREATMENT

Eight patients with clinically definite MS and with clinically active disease were studied: patients were treated with methylprednisolone, 1 g, intravenously in 250 mL of isotonic sodium chloride solution for 1 hour. The clinical features of the patients are reported in the Table. Blood samples were collected before treatment onset (T0) and after 3 and 24 hours (T3 and T24, respectively). Serum samples were stored at −80°C. Peripheral blood mononuclear cells (1 × 10^6 per milliliter) was isolated by the Ficoll-Hypaque density gradient (Pharmacia, Uppsala, Sweden) at T0, T3, and T24 and were incubated for 48 hours in RPMI with 10% fetal calf serum (at 37°C and with 5% carbon dioxide) for MMP-2 and MMP-9 quantitation. Supernatants were collected and stored at −80°C.

In separate experiments, at the same time points, PBMNCs (1 × 10^6 per milliliter) were incubated for total RNA extraction, and reverse transcriptase–polymerase chain reaction with primers specific for MMP-2 and MMP-9 was performed. Peripheral blood mononuclear cells isolated by the Ficoll-Hypaque density gradient at T0, T3, and T24 were also used for experiments of transmigration through endothelial cell monolayers, as described in the “Transmigration Assay” subsection of this section.

IN VITRO TREATMENT

Human umbilical vein endothelial cells from 6 different healthy donors, grown until confluence on type I collagen gel (Sigma-Aldrich Italia, Milan, Italy), were either unstimulated or stimulated for 24 hours with interferon-γ, 250 U/mL (Roche Italia, Monza, Italy); in the last 18 hours, methylprednisolone, 100 or 300 µg/mL (Sigma-Aldrich Italia), was or was not added to the HUVECs. The concentrations used were suggested by the concentrations found in the cerebrospinal fluid (CSF) of patients receiving intravenous methylprednisolone treatment for MS.

Supernatants were collected and stored at −80°C for MMP-2, MMP-9, and Mig quantitation. These experiments were performed to assess possible changes in interferon-γ–induced release of MMPs or Mig on incubation of HUVECs with methylprednisolone.

In separate experiments, HUVECs treated in the same way were lysed to check the expression of messenger RNA (mRNA) coding for MMP-2 and MMP-9.

TRANSMIGRATION ASSAY

The in vitro transendothelial migration of PBMNCs was studied according to the procedure described in detail by Pietschmann et al.

Briefly, endothelial monolayers were incubated with PBMNCs (1 × 10^6 per milliliter) in complete medium for 3 hours at 37°C (with 5% carbon dioxide). Three different populations of PBMNCs were recovered (nonadherent, adherent, and transmigrated cells) and quantified by optic microscopy using an indirect immunofluorescence technique (unstained anti–CD45 monoclonal antibody, followed by goat anti–mouse IgG fluorescein isothiocyanate conjugated; Becton Dickinson, Mountain View, Calif).

Transmigration With PBMNCs From Methylprednisolone-Treated Patients With MS

Human umbilical vein endothelial cells were grown on bovine type I collagen gels (Sigma-Aldrich Italia) into 16-mm wells. Before the interaction, HUVECs either were not stimulated or were stimulated for 24 hours with interferon-γ, 250 U/mL. Transmigration experiments were performed with PBMNCs isolated by the Ficoll-Hypaque density gradient at T0, T3, and T24.

Transmigration With PBMNCs From Healthy Controls

Human umbilical vein endothelial cells grown until confluence on type I collagen gels (Sigma-Aldrich Italia) were either unstimulated or stimulated for 24 hours with interferon-γ, 250 U/mL; in the last 18 hours, methylprednisolone, 100 or 300 µg/mL, was or was not added to the HUVECs.

In a previous study, a single administration of intravenous methylprednisolone, 1 g, in patients with clinically active MS reduced, after 3 hours, the ability of PBMNCs isolated from the patients to adhere to endothelial monolayers.

Herein, we evaluate if the same methylprednisolone schedule in patients with clinically active MS is able to modulate the transmigration of PBMNCs in an in vitro model.

To elucidate whether methylprednisolone treatment may also have an effect on endothelium per se, we studied the transmigration of PBMNCs from healthy control subjects through methylprednisolone-treated endothelial monolayers.

We also evaluated the possible role of MMP-9, MMP-2, and Mig in our transmigration model.

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Transmigration experiments were performed with freshly isolated PBMCs from 6 healthy controls (3 men and 3 women, aged 33-44 years).

These experiments were performed to check whether methylprednisolone treatment of the sole endothelial cells had any effect on migration of PBMCs taken from untreated healthy controls at a single time point.

**TRANSMIGRATION INHIBITION EXPERIMENTS**

T lymphocytes from one healthy control were stimulated with interleukin (IL) 2, 100 U/mL, every other day for 10 days. We performed only one transmigration experiment in triplicate with IL-2–stimulated T lymphocytes, with or without a blocking anti–Mig antibody, 10 ng/mL. (rabbit anti–Mig polyclonal antibody; PeproTech Inc, Rocky Hill, NJ); HUVECs were stimulated with interferon-γ or co-incubated with interferon-γ and methylprednisolone, 100 or 300 µg/mL, for 24 hours. This set of experiments was performed to verify if anti–Mig antibody could to some extent decrease the migration of activated cells through the endothelium.

**CXCR3 EXPRESSION**

CXCR3 expression was evaluated on T lymphocytes from one healthy control after 10 days of IL-2 treatment. The cells were stained with anti–CXCR3 fluorescein isothiocyanate conjugated (R&D Systems Inc, Minneapolis, Minn) and anti–CD3 and anti–CD14 monoclonal antibodies (Becton Dickinson) and then analyzed by flow cytometry (FacStar Plus, Becton Dickinson).

**MMP-9, MMP-2, AND Mig DOSAGE**

MMP-9 and MMP-2 were quantified in PBMC supernatants and in serum samples at T0, T3, and T24 and in HUVEC supernatants with commercially available enzyme-linked immunosorbent assay kits (R&D Systems Inc; and Oncogene, Boston, Mass, respectively).

Mig was quantified in HUVEC supernatants using a homemade sandwich enzyme-linked immunosorbent assay. Briefly, a flat-bottom 96-well plate (MaxiSorp, Nunc International, Naperville, Ill) was coated with 100 µg per well of goat anti–Mig polyclonal antibody (R&D Systems Inc), 3 µg/mL (in 0.1M carbonate buffer, pH 9.5), for 24 hours at 4°C and then washed twice with phosphate-buffered saline (PBS), pH 7.5, and washing buffer (0.05% polysorbate 20 [TWEEN 20]) and twice again with PBS alone.

Then, 250 µL per well of 5% PBS wt/vol bovine serum albumin was used to block wells for 2 hours at room temperature; 100 µL per well of Mig standards (R&D Systems Inc) or cell-derived culture supernatants was then added, followed by an overnight incubation at 4°C.

The plate was rinsed with washing buffer before the addition of 100 µL per well of rabbit anti–Mig polyclonal antibody (PeproTech Inc), 0.6 µg/mL (in PBS), and incubated at 37°C for 4 hours.

Again, the plate was rinsed with washing buffer before the addition of 100 µL per well of peroxidase-conjugated goat anti–rabbit immunoglobulins (Dako, Glostrup, Denmark), 0.125 ng/mL (in PBS, pH 7.5), and incubated for 1 hour at room temperature.

After washing 3 times, 100 µL of stabilized chromogen, tetramethylbenzidine (Sigma-Aldrich Italia), was added and left to develop for 15 minutes in the dark. The reaction was stopped with 100 µL of 1M sulfuric acid, and the absorbance was measured at 490 nm.

**REVERSE TRANSCRIPTASE–POLYMERASE CHAIN REACTION**

Total RNA was extracted using a commercially available kit (QIAGEN, Hilden, Germany) from HUVECs (as described in the “In Vitro Treatment” subsection of this section) and PBMCs (as described in the “In Vivo Treatment” subsection of this section). The complementary DNA was synthesized with Moloney murine leukemia virus reverse transcriptase and random priming. Polymerase chain reaction was performed in a buffer containing 10 mM (hydroxymethyl)aminomethane; 1.5 mM magnesium chloride; and 50 mM potassium chloride, pH 8.3) with 200 µM of each deoxyribonucleoside triphosphates and 500 µM of specific primers. The reaction consisted of 25 cycles of denaturation at 95°C for 1 minute, annealing at 55°C for 1 minute, and extension at 72°C for 1 minute. Polymerase chain reaction was performed with primers specific for glyceraldehyde-3-phosphate dehydrogenase,14 MMP-2, and MMP-9.15 Polymerase chain reaction products were electrophoresed on 1.5% agarose gel, visualized, and photographed after ethidium bromide staining.

Statistical analysis was performed using the Wilcoxon signed rank test.

**RESULTS**

In transmigration experiments performed with PBMCs from methylprednisolone-treated patients with MS, there was a significant decrease in the absolute number of transmigrating cells at T3. At T24, the number of transmigrating cells increased again, with a “rebound” effect. The pattern was similar regardless of previous stimulation of HUVECs with interferon-γ (Figure 1A and B).

Matrix metalloproteinase 2 was undetectable in PBMC supernatants, while MMP-9 was detectable only in 4 patients and became undetectable after methylprednisolone treatment, at T3 and T24 (data not shown).

Serum MMP-2 levels did not change during methylprednisolone in vivo treatment, while MMP-9 concentrations significantly increased at T24 (P < .04, Wilcoxon signed rank test). The data are as follows (levels of MMP-2 and MMP-9 are given as mean ± SD):

<table>
<thead>
<tr>
<th>Time</th>
<th>MMP-2 Level, ng/mL</th>
<th>MMP-9 Level, ng/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>T0</td>
<td>225.4 ± 61.5</td>
<td>187.5 ± 93.1</td>
</tr>
<tr>
<td>T3</td>
<td>238.3 ± 81.9</td>
<td>308.4 ± 151.9</td>
</tr>
<tr>
<td>T24</td>
<td>210.3 ± 60.4</td>
<td>373.0 ± 209.5</td>
</tr>
</tbody>
</table>

The total RNA obtained from PBMCs of patients with MS who were untreated or treated with methylprednisolone, after T3 and T24, was analyzed by reverse transcriptase–polymerase chain reaction with primers specific for MMP-9 and glyceraldehyde-3-phosphate dehydrogenase.
dehydrogenase (Figure 2A and B, respectively); a moderate increase of the mRNA coding for MMP-9 was observed in the PBMCs always at T24 (4 of the 6 patients tested).

During in vitro experiments with PBMCs from healthy controls, we found that both concentrations of methylprednisolone were able to significantly reduce the number of cells migrating through unstimulated and interferon-γ–stimulated endothelium (Figure 3A and B, respectively).

The results of the transmigration experiment performed with IL-2–stimulated T lymphocytes from one healthy control are reported in Figure 4. Incubation with anti–Mig antibody was able to partly inhibit transmigration.

Interferon-γ stimulation induced the release of Mig in HUVEC supernatants, and methylprednisolone treatment was able to reduce Mig production in a dose-dependent way, although the decrease did not reach statistical significance (Figure 5).

Matrix metalloproteinase 9 was undetectable in HUVEC supernatants and in mRNA, while MMP-2 slightly decreased with interferon-γ treatment and did not show significant (P<.60) fluctuations after methylprednisolone incubation (data not shown). However, MMP-2 mRNA at T24 showed an increase in 4 of 6 interferon-γ–stimulated HUVECs (Figure 6A). Data for GAPDH are shown in Figure 6B.

CXCR3 expression on IL-2–stimulated T lymphocytes is reported in Figure 7.

**COMMENT**

The modulation of transmigration through BBB exerted by corticosteroids is rather complex. Some corticosteroids, namely ethinyl estradiol and cyproterone acetate, reduce transmigration of leukocytes through endothelial cell monolayers after a treatment targeting endothelial cells. 16

Methylprednisolone might also influence the adhesion of PBMCs to endothelial cells: Elovaara et al17 found a reduction in the proportion of very late antigen 4–, leukocyte function–associated antigen 1–, and intercellular adhesion molecule 1–positive blood lymphocytes and monocytes during in vivo methylprednisolone treatment of patients with MS who were in relapse. Moreover, it was demonstrated that in vivo methylprednisolone therapy temporarily reduced adhesion of PBMCs to HUVECs.9

The present work shows that methylprednisolone can actually modulate transmigration through HUVEC monolayers via a direct action on the endothelium and on PBMCs.

Methylprednisolone treatment of human brain endothelial cells is able to decrease, in a dose-dependent manner, the levels of cytokine-induced intercellular adhesion molecule 1, vascular cell adhesion molecule 1, and HLA-DR.18 However, the reduction of adhesion molecule expression may not be the sole or the major mechanism leading to this biological effect. It is known that during inflammation there is production of chemokines such as Mig, inducible protein 10, and interferon-inducible T-cell alpha-chemoattractant (ITAC), which are

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**Clinical Features of the Patients**

<table>
<thead>
<tr>
<th>Patient No./ Sex/Age, y</th>
<th>Disease Duration, y</th>
<th>Disability Score†</th>
<th>Course‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>1/M/56</td>
<td>2</td>
<td>3.5</td>
<td>SP</td>
</tr>
<tr>
<td>2/F/21</td>
<td>1</td>
<td>3.0</td>
<td>RR</td>
</tr>
<tr>
<td>3/M/54</td>
<td>10</td>
<td>3.5</td>
<td>RR</td>
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</tr>
<tr>
<td>8/F/29</td>
<td>2</td>
<td>3.0</td>
<td>RR</td>
</tr>
</tbody>
</table>

*All patients were in the relapse phase of the disease.
†Calculated using the scale of Kurtzke. 11
‡SP indicates secondary progressive; RR, relapsing remitting; and RP, relapsing progressive.
molecules able to attract PBMCs carrying the CXCR3 receptor,\textsuperscript{19} and a role for these chemokines has been suggested in enhancing inflammation in patients with MS.\textsuperscript{6} In previous transmigration experiments performed with anti–ITAC, anti–inducible protein 10, or anti–Mig antibodies, the last one was the most effective in decreasing the transmigration rate of IL-2–stimulated T lymphocytes,\textsuperscript{20} suggesting that this chemokine could be more relevant than inducible protein 10 and ITAC. The addition of an anti–Mig antibody indeed decreased the transmigration rate of IL-2–stimulated T lymphocytes (Figure 4), which were mostly CXCR3\textsuperscript{+}, as shown in Figure 7, confirming Mig relevance in our transmigration model.

Our data also show that methylprednisolone treatment in vitro obtains reduction of interferon-γ–induced Mig released by HUVECs, suggesting a direct effect on endothelial cells. The reduction of Mig release paralleled the observed reduction in transmigration through the endothelial monolayer of PBMCs from healthy controls. However, the presence of anti–Mig antibody seems to have an additive effect with methylprednisolone.

As far as changes in chemokines during pharmacological treatment of patients with MS are concerned, Tarlo and coworkers\textsuperscript{21} showed that in vivo interferon beta-1b treatment in patients with MS reduced the regulated on activation, normal T cell expressed and secreted concentration, while another study\textsuperscript{22} showed that interferon beta-1a and methylprednisolone treatments induced serum and CSF fluctuations in monocyte chemoattractant protein 1 and inducible protein 10 that did not reach statistical significance. A decrease in chemokine production could be one of the beneficial effects exerted by different drugs used in MS therapy.

Our results after in vivo treatment of patients with clinically active MS with a single dose of methylprednisolone, 1 g, show that PBMCs from these patients display a marked reduction in transmigration at T3; this reduction is still short lasting, because levels went back to baseline values or even exceeded them at T24. These observations show that extravasation of PBMCs from blood to brain (which is one of the mechanisms leading to CNS damage in patients with MS) is only temporarily dampened by a single daily dose of methylprednisolone. This is of clinical relevance, because it suggests that fractionated additional doses may be needed to achieve persistent reduction in transmigration during treatment. However, our data suggest that methylprednisolone exerts its anti-inflammatory effects on endothelial and mononuclear cells. Postulating an additive effect, methylprednisolone could be even more effective in reducing transmigration phenomena in vivo.
Our data about MMP production and putative modulation by methylprednisolone in serum and PBMNCs do not seem to suggest a clear correlation with the disease and/or the pharmacological treatment.

In patients treated with methylprednisolone, we detected an increase of serum levels of MMP-9 at T24 and an increase of the expression of mRNA coding for MMP-9 in PBMNCs; on the other hand, in supernatants of cultured PBMNCs of 4 of 8 patients (the only ones who had detectable levels of MMP-9 at T0), we observed a decrease in MMP-9 production at T3 and T24.

No major changes in the release of MMP-2 were seen after HUVECs were treated with methylprednisolone. The slight increase of MMP-2 mRNA after interferon-γ stimulation was not paralleled by an increase in MMP-2 concentration in the supernatants.

In other studies, a decrease in transmigration has been related to a reduced production in MMPs. Corticosteroids may be able to modulate MMP release, but data are partially controversial. While a reduction of MMP-9 activity in the CSF of methylprednisolone-treated patients with MS after 3 days of therapy has been observed, paralleling improved BBB function, a recent study on the immunological effects of oral high-dose methylprednisolone in patients with MS did not find differences in the activity of MMP-9 in the CSF of patients a week after treatment, despite a decrease of lesions shown on the magnetic resonance imaging scan.

Most of the published data concerning the relevance of MMPs in patients with MS are mainly obtained in the CSF or in immunopathological studies.

Dexamethasone influences MMP-9 expression induced by proinflammatory cytokines in brain microvas-
circular endothelium. Our data on HUVECs, however, did not show a modulation of MMP production by methylprednisolone; 18 hours of methylprednisolone treatment is probably too short, but in our experimental model this timing did decrease transmigration.

Further studies are needed to confirm these findings and to assess combination treatment with interferon beta/copolymer-1 (glatiramer acetate) and corticosteroids with the aim of reducing PBMNC infiltration in the CNS, with a possibly additive effect, similar to what has been documented for BBB damage with methylprednisolone and interferon beta-1a.

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Author contributions: Study concept and design (Drs Bernardi, Massa, Boiardi, and Salmaggi); acquisition of data (Mr Gelati, Ms Cossini and Masini, and Dr De Rossi); analysis and interpretation of data (Mr Gelati, Ms Cossini, and Drs De Rossi and Salmaggi); drafting of the manuscript (Mr Gelati, Ms Cossini and Masini, and Drs De Rossi and Salmaggi); critical revision of the manuscript for important intellectual content (Drs Bernardi, Massa, and Boiardi); statistical expertise (Mr Gelati and Dr De Rossi); obtained funding (Drs Bernardi, Boiardi, and Salmaggi); administrative, technical, and material support (Ms Masini); and study supervision (Ms Cossini and Dr Massa).

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