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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Transmigration of peripheral blood mononuclear cells (PBMNCs) into brain parenchyma through the blood-brain barrier (BBB) plays a pivotal role in the pathogenesis of multiple sclerosis (MS).

Recent work suggests that drugs such as interferon beta-1b or glatiramer acetate, commonly used in the long-term treatment of MS, are able to modulate transmigration phenomena.

Synthetic corticosteroids, which are largely used in the treatment of MS relapses, might influence PBMNC/endothelium adhesion and/or transmigration. Corticosteroids affect leukocyte extravasation, probably by decreasing adhesion molecule expression on the vascular wall or in serum and/or by a direct action on leukocytes.

Hydrocortisone, a potent stimulator for the in vitro formation of BBB-like features, produces an increase in transendothelial resistance and a decrease in permeability for sucrose in pig brain capillary endothelial cells; moreover, we found a dose-dependent increase in electric resistance in a model of the BBB consisting of a coculture of astrocytes and endothelium grown at the opposite sides of a transwell insert after methylprednisolone hemisuccinate treatment (unpublished data, 1999).

Chemokines are proinflammatory chemotactic molecules able to selectively recruit leukocyte subsets. Transmigration of PBMNCs through the BBB in patients with...
MS is enhanced by some chemokines, such as mono-
kine induced by interferon-γ (Mig). The ability of cort-
icosteroids to decrease chemokine production is still
under investigation.

Finally, in cytokine-stimulated rat brain endothe-
lium cells, dexamethasone partially inhibits the in vitro
production of matrix metalloproteinase (MMP) 9, the high
levels of which may contribute to BBB disruption.

Magnetic resonance imaging studies have docu-
mented the reduction of BBB damage (ie, of enhance-
ment after the addition of gadolinium) obtained after in
vivo corticosteroid administration; however, it is not clear
whether this effect is mediated by an activity on circu-
lating immune cells and/or on brain microvascular en-
dotheium.

In a previous study, a single administration of in-
travenous methylprednisolone, 1 g, in patients with cli-
ничally active MS reduced, after 3 hours, the ability of
PBMCs isolated from the patients to adhere to endo-
thelial monolayers.

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

To elucidate whether methylprednisolone treatment
may also have an effect on endothelium per se, we
studied the transmigration of PBMCs from healthy con-
trol subjects through methylprednisolone-treated endo-
thelial monolayers.

We also evaluated the possible role of MMP-9, MMP-2,
and Mig in our transmigration model.
Transmigration experiments were performed with freshly isolated PBMNCs 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 PBMNCs 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 monocolonal 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 PBMNC supernatants and in serum samples at T0, T3, and T24 and in HUVEC supernatants with commercially available enzyme-linked immunosorbert assay kits (R&D Systems Inc; and Oncogene, Boston, Mass, respectively).

Mig was quantified in HUVEC supernatants using a homemade sandwich enzyme-linked immunosorbert 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 450 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 PBMNCs (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 tris(hydroxymethyl)aminomethane; 1.5 mM magnesium chloride; and 50 mM potassium chloride, pH 8.3) with 200 µM of each deoxyribonucleoside triphosphates and 500 mM 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-9.51 Polymerase chain reaction products were electrophoresed on 1.3% 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 PBMNCs 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 PBMNC 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 PBMNCs 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 PBMNCs always at T24 (4 of the 6 patients tested).

During in vitro experiments with PBMNCs 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 < .06) 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.10

Methylprednisolone might also influence the adhesion of PBMNCs 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 PBMNCs 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 PBMNCs.

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...
molecules able to attract PBMNCs carrying the CXCR3 receptor, and a role for these chemokines has been suggested in enhancing inflammation in patients with MS. 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, 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+, 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 PBMNCs 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 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 showed that interferon beta-1b 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 PBMNCs 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 PBMNCs 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-
cicular 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.27

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Author contributions: Study concept and design (Drs Bernardi, Massa, Boiardi, and Salmaggi); acquisition of data (Mr Gelati, Ms Corsini and Masini, and Dr De Rossi); analysis and interpretation of data (Mr Gelati, Ms Corsini, and Drs De Rossi and Salmaggi); drafting of the manuscript (Mr Gelati, Ms Corsini and Masini, 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 Corsini and Dr Massa).

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