

High-Dose Methylprednisolone Reduces Cytokine-Induced Adhesion Molecules on Human Brain Endothelium

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ABSTRACT: *Objective:* We investigated the *in vitro* effects of low- and high-dose methylprednisolone (MP) on the cytokine-induced expression of HLA-DR, ICAM-1 and VCAM-1 on human brain microvessel endothelial cells (HBMECs). *Methods:* Brain endothelium was obtained from microvessels included in the apparently normal white matter of surgical specimens of nine patients. Cells were stained with monoclonal antibodies anti-HLA-DR, anti-ICAM-1 and anti-VCAM-1 and analysed by flow cytometry as fluorescence histograms. The mean fluorescence intensity (MFI) of HBMECs treated with different stimuli was calculated. *Results:* γ -IFN-induced HLA-DR was down-regulated in a dose-dependent manner by MP. High-dose MP reduced the TNF- α -induced ICAM-1 and VCAM-1 expression. *Conclusions:* The down-regulation of adhesion molecules on cerebral endothelial cells could decrease mononuclear cell transmigration through the blood brain barrier and consequently the perivascular infiltrates. The results add support to the rationale for high-dose MP treatment in multiple sclerosis relapses.

RÉSUMÉ: Des doses élevées de méthylprednisolone réduisent l'expression des molécules d'adhésion induites par les cytokines sur l'endothélium de cerveaux humains. *Objectif:* Nous avons évalué les effets de faibles et de fortes doses de méthylprednisolone (MP) *in vitro* sur l'expression de HLA-DR, ICAM-1 et VCAM-1, induite par des cytokines, sur les cellules endothéliales de microvaisseaux de cerveaux humains (CEMCHs). *Méthodes:* De l'endothélium de cerveau a été obtenu de microvaisseaux provenant de substance blanche apparemment normale contenue dans des spécimens chirurgicaux provenant de huit patients. Les cellules ont été marquées au moyens d'anticorps monoclonaux anti-HLA-DR, anti-ICAM-1 et anti-VCAM-1 et analysées par cytométrie de flux sous forme d'histogramme de fluorescence. La moyenne d'intensité de la fluorescence des CEMCHs traitées au moyen de différents stimuli a été calculée. *Résultats:* Le HLA-DR induit par γ -IFN était régulé à la baisse de façon dose dépendante par la MP. De fortes doses de MP diminuaient l'expression de ICAM-1 et VCAM-1 induite par TNF- α . *Conclusions:* La régulation à la baisse des molécules d'adhésion sur les cellules endothéliales cérébrales pourrait diminuer la transmigration des cellules mononucléaires à travers la barrière hémato-encéphalique et conséquemment les infiltrats périvasculaires. Ces résultats appuient le concept d'administrer de fortes doses de MP dans le traitement de poussées de sclérose en plaques.

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Clinical trials have suggested the efficacy of high-dose methylprednisolone (MP) in the treatment of multiple sclerosis (MS) relapses,¹ while low-dose MP is not as effective and might lead to relapse reactivation in some cases.² Immunological studies focusing on the effects of steroids treatment in MS produced interesting data regarding the interface lymphomonocytes-brain endothelium.

Particularly, a reduced adhesion of lymphomonocytes to human umbilical vein endothelial cells was obtained after three hours from a single infusion of high-dose of MP (1g) in clinically active patients.³ Also, some authors report a transient down-regulation of adhesion molecules on peripheral blood mono-

nuclear cells after treatment of MS relapses with *i.v.* administration of MP,⁴ while other authors have shown that steroids are able to down-regulate the expression of some adhesion molecules on human umbilical vein endothelial cells.⁵ The reduced expression of these molecules, after steroid

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administration *in vivo*, could actually decrease the transmigration of mononuclear cells through the brain microvessels and/or the presentation of (auto)antigen at the blood brain barrier. The adhesion/transmigration process involves a number of molecular interactions,^{6,7} among which ICAM-1/LFA-1 and VCAM-1/VLA-4 have been shown to be relevant in experimental allergic encephalomyelitis.^{8,9} Interestingly, it has been suggested that Class II molecules could also be involved in lymphocyte adhesion to murine brain endothelium.¹⁰

The aim of this study was to explore if steroid treatment may target the expression of adhesion molecules on human brain microvessel endothelial cells (HBMECs). We investigated the *in vitro* effects of both low-dose MP and high-dose MP treatment on the cytokine-induced expression of HLA-DR, ICAM-1 and VCAM-1 on HBMECs.

MATERIALS AND METHODS

HBMECs were obtained from microvessels included in the apparently normal white matter of surgical specimens of nine patients (6F, 3M, age range 26-76, mean age 48.6), four of them operated for meningiomas, four operated for low-grade gliomas and one for pituitary adenoma.

After removal of leptomeninges and vessels from the surgical specimen, HBMECs were isolated by trypsinization, filtration through a 100 mesh screen and centrifugation in 15% dextran. The cells were then incubated in collagenase and plated on collagen-coated dishes. For the experiments, HBMECs were grown on 48 multiwell plates and used at the second passage. In our hands, HBMECs after the 4th passage display changes in surface amounts of ICAM-1 and VCAM-1, and also of their inducibility by cytokines (unpublished observations). The endothelial cells' morphology and HLA-DR, ICAM-1 and VCAM-1 expression in basal conditions were suggestive of normal cerebral microvessel endothelial cells, as described in the literature.^{11,12,13}

The culture purity (>95%) was tested by a von Willebrand factor (VWF) antigen staining by indirect immunofluorescence

(mouse anti-human VWF and goat anti-mouse FITC, Sigma).

Preliminary experiments were performed to evaluate the most effective stimulus in inducing HLA-DR, ICAM-1 and VCAM-1 expression on endothelium. We tested both γ -IFN and TNF- α and, while HLA-DR was induced only by γ -IFN treatment, ICAM-1 and VCAM-1 were only slightly increased by γ -IFN, with a more marked up-regulation after TNF- α incubation.

Grown cells were then studied in basal conditions and after 72 hours stimulation with the proinflammatory cytokines γ -IFN (250 U/ml) for HLA-DR induction and TNF- α (10 ng/ml) for ICAM-1 and VCAM-1 expression respectively.

In order to evaluate the most effective dose of MP in down-regulating the expression of cytokine-induced adhesion molecules, we tested five different concentrations of the drug in preliminary experiments (i.e., 65, 150, 300, 450 and 650 μ g/ml).

All nine HBMECs samples were then stimulated with "high-dose" (450 μ g/ml, i.e., the lowest dose maintaining an optimal efficacy in the down regulation of adhesion molecules) and "low-dose" MP (65 μ g/ml), alone or in combination with proinflammatory cytokines. HBMECs were ultimately stained with monoclonal antibodies (anti HLA-DR fluorescein-conjugated, Becton Dickinson, CA, anti ICAM-1 and anti VCAM-1 phycoerythrin-conjugated, Ancell Corporation, MN) and analysed by flow cytometry as fluorescence histograms. A monoclonal antibody for IgG₁ and IgG_{2a} (Simutest Control, Becton Dickinson, CA) was used to evaluate nonspecific staining. The mean fluorescence intensity (MFI) of HBMECs treated with different stimuli was calculated.

Statistical analysis of the MFI of unstimulated, cytokine-stimulated and high and low dose-MP-treated (with or without cytokine) HBMECs respectively was performed using the Wilcoxon signed rank test.

RESULTS

The results obtained are reported in the Table and Figures 1 and 2.

HLA-DR was induced only after γ -IFN stimulation but not

Table: Mean fluorescence intensity fluctuations after cytokines and MP treatment

	HLA-DR	ICAM-1	VCAM-1
Basal	n.d.*	129.8±34.4	n.d.
γ -IFN (250U/ml)	509.8±109.4 ^a	n.p.*	n.p.
TNF- α (10 ng/ml)	n.d.	1826.1±627.0 ^d	238.2±80.0 ^f
[^] MP _l + γ -IFN	430.7±115.0 ^b	n.p.	n.p.
^o MP _h + γ -IFN	240.3±87.9 ^c	n.p.	n.p.
MP _l +TNF- α	n.p.	1742.1±542.0	205.8±41.0
MP _h +TNF- α	n.p.	1408.5±457.0 ^e	113.4±28.0 ^g

* not detectable

[^]MP_l: low-dose MP

* not performed

^oMP_h: high-dose MP

Due to the minor effect of γ -IFN on ICAM-1 and VCAM-1 expression, observed in preliminary experiments, these data are omitted in the table.

Wilcoxon signed Rank Test: a-b p<0.015; a-c p<0.008; d-e p<0.015; f-g p<0.011

(Data obtained in nine individuals. In five of these, the experiments were run in duplicate, with no differences between the observed values. Only one value for each individual was retained in the statistical evaluation).

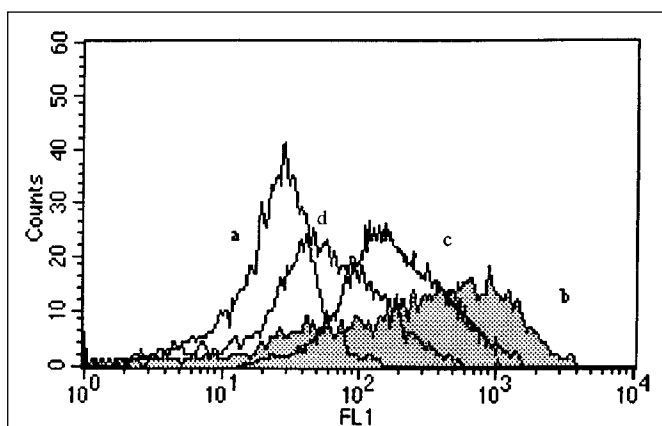


Figure 1a: Down-regulation of γ -IFN-induced HLA-DR by high-dose MP. The x axis reports the fluorescence 1 values (FL1), while the y axis reports the number of cells. (a) Simulstest Control-marked HBMECs (b) γ -IFN stimulated HBMECs (filled gray histogram) (c) γ -IFN+150 μ g/mL-MP-stimulated HBMECs (d) γ -IFN+high-dose MP-stimulated HBMECs

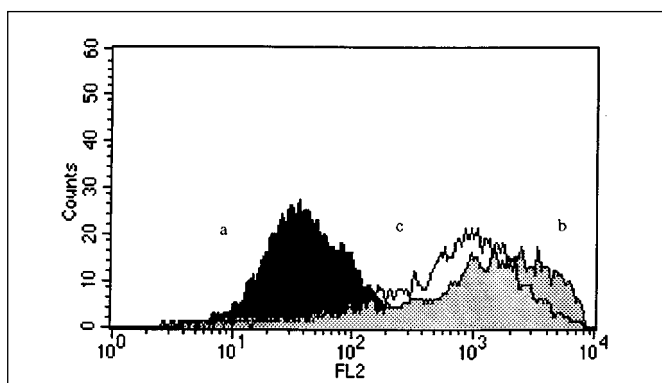


Figure 1b: Down-regulation of TNF- α -induced ICAM-1 by high-dose MP. The x axis reports the fluorescence 2 values (FL2), while the y axis reports the number of cells. (a) Basal ICAM-1 expression on HBMECs (filled black histogram) (b) TNF- α -stimulated HBMECs (filled gray histogram) (c) TNF- α +high-dose MP-stimulated HBMECs

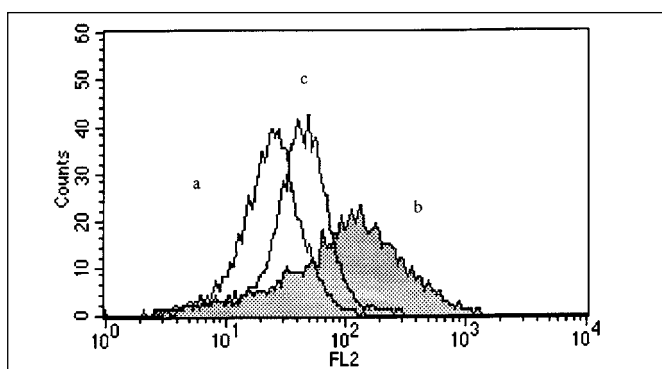


Figure 1c: Down-regulation of TNF- α -induced VCAM-1 by high-dose MP. The x axis reports the fluorescence 2 values (FL2), while the y axis reports the number of cells. (a) Simulstest Control-marked HBMECs (b) TNF- α -stimulated HBMECs (filled gray histogram) (c) TNF- α +high-dose MP-stimulated HBMECs

after TNF- α stimulation; the induced expression was down-regulated in a dose-dependent manner by MP (Figure 1a and Table).

ICAM-1 was expressed constitutively and increased after stimulation by TNF- α : high-dose MP reduced the induced expression significantly (Figure 1b and the Table); the intermediate concentration of 300 μ g/ml was as effective as the highest (data not shown). Low-dose MP was ineffective in counteracting TNF- α -induced ICAM-1 expression (Table). Both high and low-dose MP alone did not modify the constitutive expression of ICAM-1 nor were able to induce expression of HLA-DR or VCAM-1 (unshown). VCAM-1 was not expressed in basal conditions (percentage of positive cells <10%), while it was induced only after stimulation with TNF- α : the TNF- α -induced expression was reduced significantly with high-dose MP (Figure 1c and the Table); the 300 μ g/ml concentration was also able to decrease TNF- α -induced VCAM-1 expression to a similar extent, whereas this was not the case for low-dose MP (Table).

DISCUSSION

In comparison with our previous results¹⁴ on human umbilical vein endothelial cells (where only a minimal decrease in γ -IFN-induced HLA-DR was detected with high-dose MP), in the present report high-dose MP was indeed effective in reducing γ -IFN-induced expression on HBMECs: this might be due either to district-specific differences and/or to the different passages of endothelial cells. While the reduction in MFI observed in cytokine-induced HLA-DR and VCAM-1 expression after high-dose MP incubation seems to be biologically relevant (the values are reduced to the half), the biological significance of ICAM-1 down-regulation is doubtful, since the MP-reduced values are still near to those induced by TNF- α . Similar results have been found in an *in vivo* study in patients with rheumatoid arthritis, in which a pulse of 1 g of MP induced a marked decrease in E-selectin and a smaller reduction in ICAM-1 expression on synovial vascular endothelium.¹⁵ In this respect, it must be stressed that *in vivo* infusion of a single dose of 1500 mg of MP,

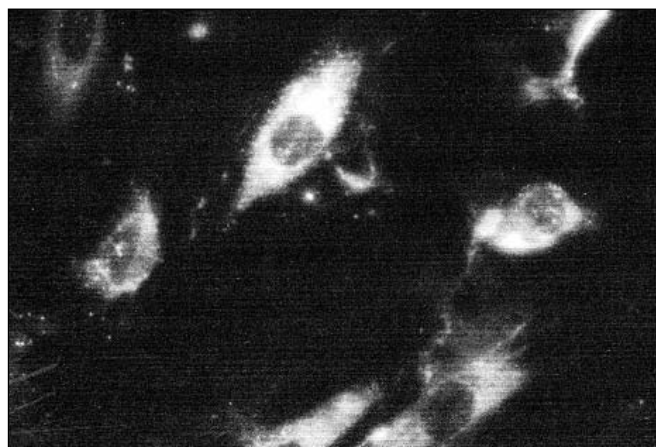


Figure 2: Human brain endothelial cell von Willebrand factor (VWF) antigen staining by indirect immunofluorescence (mouse anti-human VWF and goat anti-mouse FITC, Sigma).

i.v., in MS patients, induced peak plasmatic concentration of the drug close to those used in our work.¹⁶

In conclusion, our data show that the immunomodulatory effects of steroids, already described on endothelium from other tissues^{14,15,17} may target also HBMECs and this is actually complementary to the similar down-regulation induced on circulating immune cells, as mentioned above.⁴ The finding gets relevance particularly when put in synergistic combination with β -IFN treatment, which, in turn, is able to down-regulate γ -IFN-induced HLA-DR expression on the surface of HBMECs,¹⁸ as well as to reduce *in vitro* transendothelial migration of TNF- α - or γ -IFN-activated leukocytes.¹⁹

A recent study has actually shown that MP treatment during relapse in β -IFN-treated MS patients produces a persistent reduction of enhancement in MRI-evaluated lesions, suggesting that β -IFN could in some way prolong the beneficial effects of steroids on the blood brain barrier.²⁰ This could be due to a downregulation of HLA-DR and adhesion molecules in combination with a decrease of MMP-9 production, observed both in β -IFN²¹ and in steroid treatment.²²

Overall, the *in vitro* results obtained in this study add some rationale to treatment of MS relapses with high-dose MP; further studies are needed to better delineate possible interactions of MP with drugs such as β -IFN able to modulate long-term disease activity.

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