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Research

Sera from Patients with Multiple Sclerosis in Relapse or Remission Affect the Blood-Brain Barrier Differently: An *In vitro* Study

Manik Mathur[1], Bruno Gran[2], Radu Tanasescu[2], Cris S. Constantinescu[1,2,3] and Ulvi Bayraktutan[1]*

[1]*Academic Unit of Mental Health and Clinical Neurosciences, School of Medicine, University of Nottingham, Nottingham, UK*

[2]*Department of Neurology, Nottingham University Hospitals NHS Trust, Nottingham, UK*

[3]*Department of Neurology, Cooper University, New Jersey, USA*

***Corresponding Author:** Dr. Ulvi Bayraktutan, Academic Unit of Mental Health and Clinical Neurosciences, School of Medicine, University of Nottingham, Nottingham, UK.*

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Abstract

Background: Breakdown of the blood-brain barrier (BBB) constitutes a key step in the pathogenesis of multiple sclerosis (MS).

Aims: To investigate whether sera from MS patients in relapse or in remission may differently affect the BBB function and to assesses the putative barrier-restorative effects of major molecular mechanisms known to regulate BBB function.

Methods: Sera were prepared by centrifugation of the whole blood samples of the study participants. A cell culture model of human BBB, consisting of human brain microvascular endothelial cells, astrocytes and pericytes, was established using transwell inserts. The integrity and function of BBB were studied by measurements of transendothelial electrical resistance (TEER) and paracellular flux of Evan's blue-labelled albumin (EBA), respectively.

Results: Sera from MS patients in relapse possessed greater levels of inflammatory cytokines (TNF-α and IL-1 β), apoptotic enzyme activity (caspase-3/7) and were more disruptive of BBB as evidenced by significant decreases in TEER and increases in EBA flux. Suppression of intracellular availability of reactive oxygen species or NADPH oxidase, Rho-kinase, protein kinase C- β and matrix metalloproteinase-2 activity by specific inhibitors markedly attenuated the BBB-disruptive effects of sera obtained from MS patients in relapse or remission.

Conclusions: A plethora of mechanisms affecting the overall status of oxidative stress, inflammation, cell viability and basement membrane integrity appear to contribute to the BBB damage in MS patients, especially those in relapse. Effective inhibition of the key elements associated with these mechanisms mitigate the deleterious effects of MS patients' sera on BBB integrity and function.

***Keywords:** blood-brain barrier; multiple sclerosis; inflammatory cytokines; apoptosis; rho-kinase; protein kinase C*

Highlights

- Exposure to sera obtained from MS patients in relapse or in remission compromise the integrity and function of an *in vitro* model of human blood-brain barrier (BBB).
- Sera from MS patients in relapse possessed greater levels of inflammatory cytokines, apoptotic enzyme activity and were more disruptive of BBB.
- Specific targeting of NADPH oxidase, MMP-2, Rho-kinase and oxidative stress markedly attenuate barrier-disruptive effects of MS patients' sera.

Introduction

Multiple sclerosis (MS) is a chronic and progressive inflammatory disorder of the central nervous system (CNS). It is characterised by relapsing and progressive clinical neurological deficits, and pathologically by neuroinflammation, demyelination, neurodegeneration, and axonal loss. The breakdown of the BBB and ensuing transmigration of blood constituents into brain parenchyma are regarded amongst the key steps in MS pathogenesis (1). Excessive release of inflammatory cytokines e.g., TNF- α and IL-1 β and subsequent dissolution of inter-endothelial cell tight junctions (TJ) are regarded amongst the main pathological processes that account for BBB breakdown (2). Besides immune aggression from the periphery, MS-derived brain microvascular endothelial cell-like cells display an inflammatory phenotype, with increased adhesion molecule expression and immune cell interactions (3). Accumulation and activation of leukocytes in brain parenchyma lead to overproduction of reactive oxygen species (ROS), a hallmark of oxidative stress whereby further augment BBB damage and exacerbate demyelination and axonal loss (4). In addition to infiltrating macrophages and microglia, endothelial cells may also contribute to exaggerated synthesis of ROS in MS patients. Despite availability of evidence showing the involvement of endothelial NADPH oxidase in TNF- α-mediated BBB disruption, its relevance to BBB damage needs scrutiny in the context of MS (5,6).

Considering the close correlation between BBB damage and MS pathogenesis, it is reasonable to think that preservation of BBB function may be of significant therapeutic importance, at least, in the initial phases of MS. Although restoration of the BBB or the modulation of the inflammatory cytokine release or oxidative stress would inevitably be of value in this context, the putative involvement of other mechanisms capable of mediating breakdown or repair of the BBB cannot be ruled out. Bearing these in mind, this study has first assessed the presence and levels of major pro-inflammatory cytokines (TNF-α and IL-1β) in serum samples of MS patients in relapse and remission. Then, it has investigated whether and how sera from patients in relapse or remission affect the integrity and function of an *in vitro* model of human BBB. Finally, it has explored whether specific targeting of intracellular ROS, NADPH oxidase, protein kinase C-β Rho kinase and matrix metalloproteinase-2 (MMP-2), can mitigate the putative deleterious effects of MS patients' sera on BBB.

Material and Methods

Human Blood Serum Samples

Sera were obtained by centrifugation of the blood samples (~15 ml) collected from healthy volunteers (HVs) or MS patients in relapse or in remission. Six different individuals, both men and women between 23-45 years of age, were recruited for each study group. Written informed consent was obtained from all participants. Sera were aliquoted into 1 ml Eppendorf tubes and stored at -80°C. Samples were collected under the ethical approval of Nottingham Research Ethics Committee 2 (MREC reference: 08/H0408/167).

In vitro model of human blood-brain barrier

A triple cell culture model of human BBB, consisting of human brain microvascular endothelial cells (BMEC), astrocytes and pericytes, was established as before (5,6) prior to incubation with serum samples of HVs or MS patients for 6 h. The integrity and function of the BBB were studied as before by measurements of transendothelial electrical resistance and paracellular flux of a permeability marker, EBA (67 kDa), respectively (5).

Similar experiments were repeated in the presence of agents targeting NADPH oxidase (apocynin, 1 mM), intracellular availability of ROS (via a cell-permeable superoxide dismutase mimetic called manganese (III) tetrakis (4-benzoic acid) porphyrin or MnTBAP, 2.5 μM), mitochondrial complex I (rotenone, 2 μM), Rho kinase (Y27632, 2.5 μM), protein kinase C-β (LY333531, 0.05 μM) and matrix metalloproteinase-2 (MMP2 inhibitor III, 100 μM).

Caspase-3/7 assay

Caspase-3/7 activities were assessed using Apo-ONE homogeneous caspase-3/7 kit (Promega; Southampton, UK) as per the manufacturer's instructions.

Measurement of inflammatory cytokine levels

The levels of pro-inflammatory cytokines TNF-α and IL-1β were measured in HV and MS sera using human TNF-alpha DuoSet DY210 ELISA and human IL-1 beta/IL-1F2 DuoSet DY008 ELISA kits, respectively as per the manufacturer's instructions (R&D systems, Abingdon, UK).

Results

Serum levels of inflammatory cytokines in healthy volunteers and MS patients

The levels of pro-inflammatory cytokines, TNF-α and IL-1β appeared to be significantly higher in sera of MS patients compared to those of HVs ($n=6$, $p<0.05$).

In this small number of samples, although the levels of both cytokines were higher in samples from MS patients in relapse than in remission, the differences did not reach significance (Fig 1).

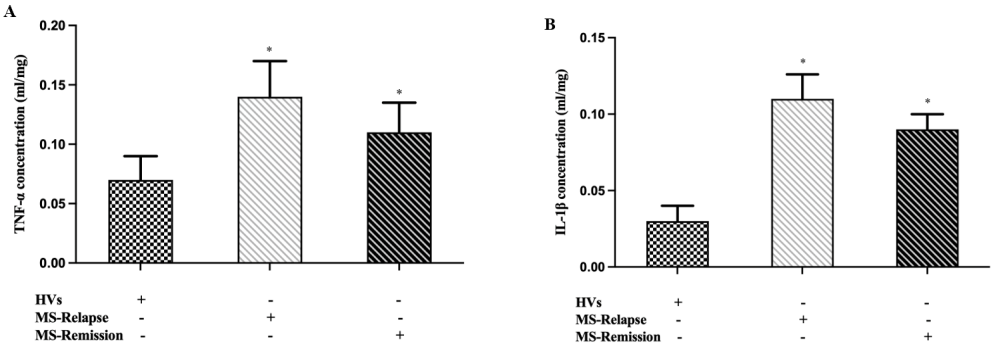

Figure 1. *The levels of pro-inflammatory cytokine TNF-α and IL-1β in healthy volunteers and MS patients in relapse or remission. Sera from both MS patient groups possess higher levels of TNF-α (A) and IL-1β (B). n=6 for each group. *p<0.05 vs controls.*

Serum caspase-3/7 activity in healthy volunteers and MS patients

The levels of serum caspase-3/7 activities were significantly higher in MS patients in relapse compared to HVs and MS patients in remission (n=6, p<0.05). No significant difference was observed in caspase-3/7 activities between HVs and patients in remission (Fig 2).

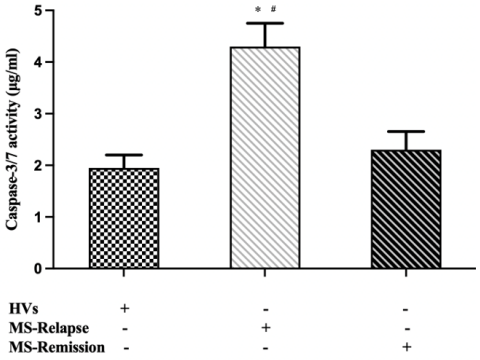

Figure 2. *The level of apoptotic caspase-3/7 activity in healthy volunteers and MS patients in relapse or remission. Sera from MS patients in relapse possess greater caspase-3/7 activity compared to those from MS patients in remission and healthy volunteers (controls). n=6 for each group. *p<0.05 vs controls. # p<0.05 vs MS patients in remission.*

The impact of MS patients' sera on BBB integrity and function

Exposure of the experimental model of human BBB to MS patients' sera led to significant disruptions in barrier integrity and function as evidenced by marked decreases in TEER and concurrent increases in EBA flux, compared to their counterparts exposed to sera from HV. The magnitude of BBB damage was considerably greater with sera from patients in relapse than in remission (n=6, p<0.05).

Specific targeting of elements contributing to overall oxidative stress showed that suppression of NADPH oxidase enzyme system or the intracellular ROS availability markedly attenuated the deleterious effects of sera of patients in relapse on BBB integrity and function (n=6, p<0.05). In contrast, treatments with apocynin (NADPH oxidase inhibitor) and MnTBAP (a SOD mimetic) only significantly improved barrier function, ascertained by reduced EBA flux, when subjected to sera of patients in remission. In contrast, inhibition of mitochondrial complex I with rotenone did not affect BBB integrity and function compared to respective sera only-treated counterparts (Fig 3A-B).

Furthermore, specific targeting of other pathways known to have relevance to BBB functionality showed that inhibition of Rho-kinase, PKC-β and MMP-2 significantly diminished the deleterious effects of sera of MS patients in relapse or in remission on BBB, where the magnitude of improvements appeared to be the least in response to LY333531, a PKC-β inhibitor (Fig 4A-B).

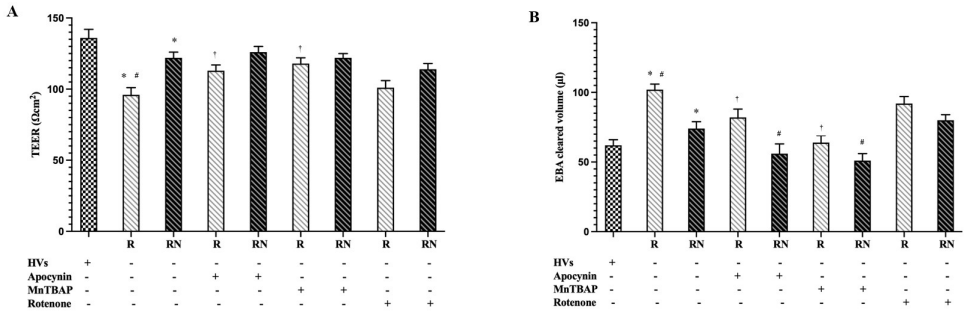

Figure 3. *The effect of sera from healthy volunteers (HVs) and MS patients in relapse or remission on blood-brain barrier (BBB) integrity and function. Sera from MS patients compromised the integrity (A) and function (B) of BBB compared to those exposed to sera of healthy volunteers (HVs). Targeting of NADPH oxidase activity (via apocynin) and intracellular availability of reactive oxygen species (via MnTBAP) differently affected BBB integrity and function. Inhibition of mitochondrial complex I by rotenone failed to affect BBB characteristics in all experimental settings. The data are presented from 6 independent experiments. *p<0.05 vs controls, †p<0.05 vs MS patients in relapse, # p<0.05 vs MS patients in remission. R: relapse, RN: remission.*

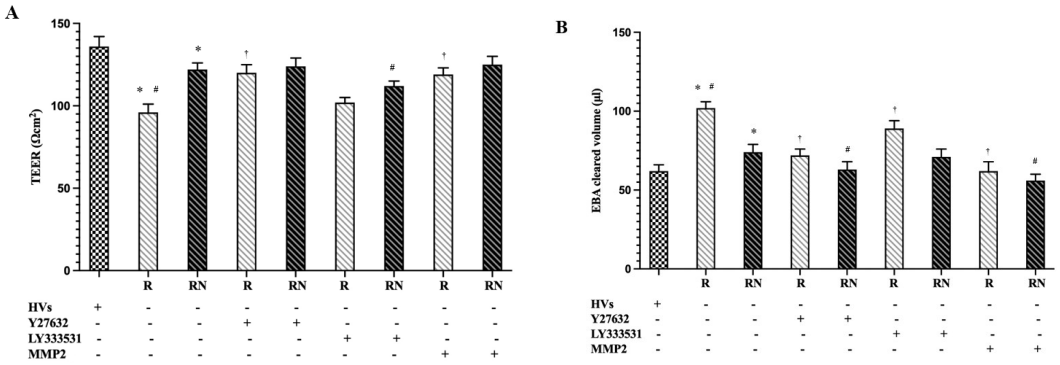

Figure 4. *The effect of sera from healthy volunteers and MS patients in relapse or remission on blood-brain barrier (BBB) integrity and function in the absence or presence of inhibitors targeting Rho-kinase, protein kinase C-β and matrix metalloproteinase-2 (MMP-2). Sera from MS patients perturbed the integrity (A) and function (B) of BBB compared to those exposed to sera of healthy volunteers (HVs). Co-treatments with Y27632 (Rho-kinase inhibitor), LY333531 (protein kinase C-β inhibitor) and MMP-2 inhibitor I (MMP-2 inhibitor) markedly diminished the barrier-disruptive effects of sera obtained from MS patients in relapse or in remission. The data are presented from 6 independent experiments. *p<0.05 vs controls, †p<0.05 vs MS patients in relapse, # p<0.05 vs MS patients in remission. R: relapse, RN: remission.*

Discussion

MS is an immune-mediated inflammatory disease that attacks myelinated axons in the CNS and is classified into subtypes on the basis of clinical course. Relapsing-remitting MS represents the most common subtype of disease and is characterised by clearly defined attacks (relapses) followed by periods of partial or complete recovery (remissions) (7). Several elements, notably pro-inflammatory cytokines TNF-α and IL-1β are known to mediate neuroinflammation in MS (8). In support of this notion, the present study shows increases and decreases in serum levels of these cytokines during active disease (relapse) and remission, respectively. Furthermore, this study also shows a strong correlation between the level of inflammatory cytokines and the extent of BBB damage as evidenced by greater decreases in TEER and increases in EBA flux across an *in vitro* model of human BBB exposed to sera of MS patients in relapse compared to those in remission. BBB damage constitutes an early event in MS pathogenesis and is in part explained by dissolution of inter-endothelial cell tight junctions (1). Given the widespread expression of cytokine receptors e.g. TNF receptor-1 and IL1-receptor 1 on cerebral microvascular endothelial cells, increased release of cytokines in MS continues to be an important issue for therapeutic purposes (9,10).

Inflammatory cytokines also trigger the apoptosis of the cells that make up the neurovascular unit (NVU) whereby they compromise the BBB. Indeed, increased levels of TNF-α have been shown to promote BBB dysfunction in part through induction of brain microvascular endothelial cell apoptosis (5,6). In accordance with these findings, analysis of caspase-3/7 enzyme activity levels in HV and MS patient sera in this study has shown substantial increases only in patients in relapse compared to HVs thereby implying the contribution of this phenomenon to BBB damage in the setting of active disease. It is noteworthy that other components of the NVU are also affected by MS in which focal or global hypoperfusion play a role. These phenomena affect contractility and viability of pericytes and ultimately promote BBB damage (11).

In light of our recent studies proving NADPH oxidase as the main enzymatic source of oxidative stress in experimental conditions mimicking excessive availability of inflammatory cytokines (5,6), it is safe to suggest that oxidative stress may play a role in MS-mediated BBB damage. Indeed, attenuation of BBB-disruptive effects of sera from patient in relapse or in remission with agents targeting this particular enzyme system and intracellular ROS confirm the accuracy of this hypothesis. Protection of endothelial cells, astrocytes and pericytes from apoptosis by MnTBAP, a well-known inhibitor of neuronal apoptosis, may also be involved in its barrier-protective effects (12). Contrary to benefits achieved with apocynin and MnTBAP, treatments with a mitochondrial complex I inhibitor, rotenone, did not have any impact on BBB characteristics compared to sera-only treated counterparts. As rotenone suppresses inflammatory responses in hepatocytes and neuroblastoma SH-SY5Y cells (13,14), differences in the modulation of inflammatory cytokines are unlikely to be involved.

The activation of PKC may also potentiate NADPH oxidase-mediated production of ROS in MS patients in relapse or in remission. Activation of MMP-2, plasminogen-plasmin system components alongside NADPH oxidase appear to account for the barrier-disruptive effects of different PKC isoforms, notably PKC-α and PKC-β in inflammatory and hyperglycaemic settings (6,15). In concordance with these studies, inhibition of PKC-β with LY333531 in the present study also diminished the BBB-disruptive effects of serum samples of MS patients in relapse or remission. In addition to BBB damage, increased expression of PKC isoforms, such as that of PKC-δ has also been proposed as an important component of progressive neurodegeneration and functional decline in MS (16).

MMP activity in MS tissues is determined by the balance between MMPs and their tissue inhibitors called TIMPs. While MMP-9 predominates in acute MS lesions and is inhibited by TIMP-1, MMP-2 appears to participate more in remodelling of the extracellular matrix (ECM) in chronic phases of the disease and is inhibited by TIMP-2. It is likely that these differences are reflected by their serum levels (17). Considering these and the seminal role of MMP-2 in TNF-α-mediated BBB breakdown (6,18), this study examined whether neutralisation of this particular isoform may influence BBB characteristics differently in the presence of sera from patients in relapse or remission. Unlike their abovementioned distinct effects at tissue level, inhibition of MMP-2 led to significant improvements in barrier integrity and function in both cases.

Amongst novel prospective treatment options for inflammatory neurodegenerative diseases, Rho-kinase attracts attention due to its crucial roles in triggering neuropathological changes and associated clinical symptoms. Neurovascular benefits realised by inhibition of Rho-kinase in an animal model of MS i.e. experimental autoimmune encephalomyelitis specifically substantiate the role of Rho-kinase in MS (19). Mitigation of the detrimental effects of sera of MS patients in relapse or remission on BBB by a specific Rho-kinase inhibitor (Y27632) further substantiate the involvement of this enzyme in MS-related neurovascular damage.

There are some limitations to this study. Even though, we have previously shown that inflammatory cytokines, notably TNF-α induces MMP-2 expression and activity in human brain microvascular endothelial cells and astrocytes (6,18), it would have been useful to study MMP-2 activity in HV and MS patients' sera to correlate these with the extent of BBB damage. Given that some differences between TEER readings and EBA flux do not correlate, it would have been useful to study the paracellular flux of a smaller size permeability marker as well to have an idea as to size of inter-endothelial cell openings before and after treatments with agents targeting different pathways.

Conclusions

The present study demonstrates that excessive release of inflammatory cytokines, metalloproteases and apoptotic enzymes caspase-3/7 during the active phase of MS may play an instrumental role in compromising the BBB which enable infiltration of leukocytes into CNS and as a result further augment demyelination and axonal loss. Hence, preservation of the BBB integrity and function is regarded as a crucial therapeutic target for MS. Our attempts to negate the deleterious effects of serum obtained from patients with MS in relapse and remission identify NADPH oxidase, MMP-2, Rho-kinase and PKC--β as potential therapeutic targets.

Conflict of Interests

The authors have no conflict of interests

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Credit Author Statement

MM performed experiments, analysed data and wrote the first draft of the paper. BG, RT and CSC recruited both the patients and HVs for the study and edited the manuscript. UB designed and supervised the study, interpreted the data, and wrote the manuscript. All authors have approved the final version of the publication.

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