Kinin B₁ Receptor Expression on Multiple Sclerosis Mononuclear Cells

Correlation With Magnetic Resonance Imaging T2-Weighted Lesion Volume and Clinical Disability

Alexandre Prat, MD, PhD, FRCPC; Katarzyna Biernacki, PhD; Tanya Saroli, MD; John E. Orav, PhD; Charles R. G. Guttmann, MD; Howard L. Weiner, MD; Samia J. Khoury, MD; Jack P. Antel, MD

Background: We have previously shown that the inducible kinin B₁ receptor is expressed on T lymphocytes during relapses and progression in multiple sclerosis.

Objective: To evaluate the correlation between the expression of B₁ receptor on peripheral blood mononuclear cells derived from patients who have multiple sclerosis with serial, clinical magnetic resonance imaging and immunological study–derived measures.

Design: Using frozen samples obtained from a high-frequency magnetic resonance imaging–immunological study, we analyzed B₁ receptor messenger RNA (mRNA) expression in peripheral blood–derived mononuclear cells serially collected from 6 patients with multiple sclerosis and 8 healthy control subjects by semiquantitative radioactive duplex reverse transcriptase–polymerase chain reaction amplification. Time-course kinin B₁–actin mRNA ratios were subsequently compared with corresponding clinical magnetic resonance imaging and immune parameters.

Results: The time-course kinin B₁–actin mRNA ratio correlated positively with the Expanded Disability Status Scale index (P<.001), occurrence of clinical relapse (P=.02), volume of lesion on T2-weighted images (P<.003) and interleukin 2 receptor and major histocompatibility complex class II expression on CD4⁺ lymphocytes, but not with gadolinium-enhancing lesions. The time-course kinin B₁–actin mRNA ratios were 5 to 25 times lower in samples derived from healthy controls.

Conclusion: The correlation of kinin B₁ receptor mRNA levels with dynamic clinical and magnetic resonance imaging measures suggests that expression of this receptor can serve as an index of disease activity in multiple sclerosis.

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Multiple sclerosis (MS) is an inflammatory demyelinating disease of the central nervous system (CNS) characterized by development of multifocal lesions disseminated in time and space. Disease activity is measured by the clinical indexes of relapse rate or change in disability index (Expanded Disability Status Scale [EDSS]) and by magnetic resonance imaging (MRI)–based indexes of new T2-weighted lesion formation on serial MRI scanning or the presence of gadolinium (Gd)-enhancing lesions. The search for biological markers that will correlate with these indexes continues.

Bradykinin, a member of the vasoactive kinin peptide family, is a modulator of the inflammatory response. Kinins exert their action by 2 distinct receptors, namely, B₁ and B₂ receptors. In contrast to the B₂ receptor, the B₁ receptor is not expressed on human cells under normal physiological conditions but can be up-regulated in situ following tissue injury and in vitro with proinflammatory cytokines. Serum levels of the endogenous B₁ receptor ligand desArg⁹-bradykinin are increased during active disease in experimental allergic encephalomyelitis, the most frequently used animal model of MS. In a cross-sectional study, we found that B₁ receptor messenger RNA (mRNA) transcripts and protein were significantly up-regulated on circulating lymphocytes during episodes of active disease in relapsing-remitting (RR) MS and secondary pro-

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MRI, lesion volume measurements, and immunological activation markers

Imaging was performed using a 1.5-T scanner (Siemens, General Electric Co, Milwaukee, Wis). T2-weighted images, T1-weighted spin-echo pulse sequence (with intravenous administration of gadolinium–diethylenetriamine-pentaacetic acid enhancement) acquisition, and computerized image analysis were done according to a protocol described previously. Expression of CD26, IL-2R, and MHC class II on the surface of PBMCs and CD4+ cells was assessed as previously described.

RNA extraction, complementary DNA synthesis, and semiquantitative duplex PCR amplification

Cryopreserved PBMCs were obtained from the Brigham and Women’s Hospital, Boston, Mass, with coded labels. Blinded analysis of B1 mRNA expression was performed in Montreal, Quebec, as described. Cells were thawed, spun down at 1500 rpm for 15 minutes, and lysed in a Trizol LS reagent (Gibco BRL, Burlington, Ontario). The RNA was extracted according to the protocol provided with Trizol. Radioactive duplex RTPCR was performed as described previously. Samples with less than 3 µg of RNA were not used in this semiquantitative RT-PCR reaction analysis. Glyceraldehyde-3-phosphate dehydrogenase primers for the PCR amplification have been substituted by actin primers since the latter were designed to span 1 intron (actin 5′ ATC TGG CAC CAC ACC TTC TAC AAT GAG, actin 3′ CGT CAT ACT CCT GCT TGC TGA TCC ACA TCT GC). Human kinin B1 receptor mRNA sequence (accession No. U12512) was used to design 5′ and 3′ primers using the Primer 3 Output software (B1 5′ primer ATC TGG TGT TTG TCT TGG TGT CTC ACC TCA GCC; B1 3′ primer AGG CCA GGA TGT GGT AGT TG; predicted amplified PCR fragment 435 base pair). The PCR reaction mix was placed in a thermal cycler (PTC-100; MJ Research Inc, Waltham, Mass) for 30 cycles at 94°C (1 minute), 62°C (45 seconds), and 72°C (1 minute). A titration curve, generated for both actin and B1 primers, revealed that 30 cycles at optimal since it is in the linear range of PCR amplification and showed no saturation. Following amplification, 20 µL of each sample was electrophoresed on a 1.5% agarose gel (Gibco BRL). Quantitative analysis of B1 and actin mRNA levels was performed using a Storm-860 PhosphoImager (Molecular Dynamics, Sunnyvale, Calif) and the ImageQuant software (Molecular Dynamic). To compare the level of B1 receptor mRNA expression, we calculated the ratio of kinin B1 to actin Phospholipids for each sample. All PCR reactions were performed in duplicate to ensure reliability of PCR amplification. The PBMC samples were not stored in a buffer that allowed protein purification after thawing and B1 receptor expression could not be analyzed at the protein level.

STATISTICAL ANALYSIS

Repeated measures linear regression was performed to determine correlation between the kinin B1–actin ratio and EDSS index, occurrence of attacks, T2-weighted lesion volume, and the number of Gd-enhancing lesions. The model used the kinin B1–actin ratio as the independent predictor and each of the clinical MRI measures separately as dependent outcome variables. The model was used across all 6 subjects, using all available time points for each subject. Because of the potential dependence between serial measurements on each subject, the model was adjusted for correlation that was assumed to diminish as the interval between serial measurements increased. Simi-

**PATIENTS**

Sufficient mRNA to perform reverse transcriptase-polymerase chain reaction (RT-PCR) in a reliable manner could be extracted from cryopreserved PBMC samples of 6 of 10 patients with MS and 8 of 12 controls randomly selected from the overall pool of 40 patients recruited during a high-frequency MRI-immunological correlational study. The details of the study have been previously reported. The 6 patients all had RR disease. The mean ± SE age of the participants was 35 ± 2.6 years (age range, 29-45 years), male-female ratio was 1:5, and a mean EDSS index at the start of the study was 4.3 (range, 2-6). Nine to 10 RNA samples were available for each patient; a total of 58 samples were analyzed. Patients selected for this study did not receive steroid therapy during the course of the study and for at least 2 months before enrollment.

**METHODS**

**Table. Comparison and Correlation of Patients With Multiple Sclerosis**

<table>
<thead>
<tr>
<th>Outcome</th>
<th>Kinin B1–Actin Ratio</th>
<th>Change in Kinin B1–Actin Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Value</td>
<td>Value</td>
</tr>
<tr>
<td></td>
<td>P</td>
<td>Value</td>
</tr>
<tr>
<td>EDSS index</td>
<td>0.22 (.02)</td>
<td>-0.001 (.10)</td>
</tr>
<tr>
<td>Occurrence of an attack, OR</td>
<td>2.8</td>
<td>1.3</td>
</tr>
<tr>
<td>T2-weighted volume, mm²</td>
<td>371 (123)</td>
<td>.003</td>
</tr>
<tr>
<td>Total No. of Gd-enhanced lesions</td>
<td>0.03 (.24)</td>
<td>.90</td>
</tr>
<tr>
<td>New Gd-enhanced lesions</td>
<td>0.02 (0.16)</td>
<td>.90</td>
</tr>
<tr>
<td>%CD4+ CD25+</td>
<td>30.4 (15.2)</td>
<td>.046</td>
</tr>
<tr>
<td>%CD3+ CD26+</td>
<td>46.4 (23.5)</td>
<td>.048</td>
</tr>
<tr>
<td>%CD4+ MHC II</td>
<td>61.6 (29.3)</td>
<td>.04</td>
</tr>
<tr>
<td>%CD3+ MHC II</td>
<td>117 (55)</td>
<td>.03</td>
</tr>
</tbody>
</table>

Abbreviations: EDSS, Expanded Disability Status Scale; Gd, gadolinium; MHC II, major histocompatibility complex class II; OR, odds ratio.

*Data are given as the effect estimate (±SE) unless otherwise indicated.

When using changes in the kinin B1–actin as the predictor, the analogous changes in outcome were analyzed.

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Table of contents: MRI, lesion volume measurements, and immunological activation markers
cularly, each kinin B₁–actin value was compared with the previous value to calculate the change in the kinin B₁–actin ratio for each patient. The analogous simultaneous change was calculated for the EDSS index, T2-weighted lesion volume and Gd-enhancing lesions. All analyses were based on actual changes, retaining information on whether the changes were positive or negative. The same repeated-measures analysis of variance model as above was then performed to determine if the predictor change in the kinin B₁–actin ratio was associated with a change in any of the clinical or MRI measures.

RESULTS

The Table summarizes the overall relationship between the absolute values (kinin B₁–actin ratio) and changes in B₁ receptor mRNA derived from PBMCs of patients who have MS with clinical, MRI, and immunological measurements. The effect estimate (±SE) and P value for each comparison are provided. Absolute values of the kinin B₁–actin ratio correlated with presence of clinical attacks (P = .024) and EDSS index (P < .001), T2-weighted lesion volume (P = .003), but not Gd-enhancing lesions, and percentage of IL-2Rα + and MHC class II CD4⁺ lymphocytes (P = .046 and P = .04, respectively) and percentage of CD26⁺ or MHC class II PBMCs (P = .048 and P = .03, respectively). Changes in B₁ mRNA expression (changes in kinin B₁–actin ratios) correlated with the presence of a clinical relapse (P = .03) but not with changes in the EDSS index, and with changes in the T2-weighted lesion volume (P = .003) but not with the presence of Gd-enhancing lesions.

Figure 1 shows the time-course expression of kinin B₁ receptor mRNA (kinin B₁–actin ratio) with corresponding EDSS index for each patient studied. We could observe preceding and/or simultaneous increase in the kinin B₁–actin ratio and EDSS index in 4 of 6 patients (patients 1-4). For these 4 patients, the increase in the kinin B₁–actin ratio was transient and B₁ levels returned to baseline. In the other patients (patients 5 and 6), the EDSS index was high (≥6) when the study was initiated and remained stable during the course of the study. When kinin B₁–actin ratios were compared with the T2-weighted lesion volume (expressed in cubic millimeters) (Figure 2), increases in this ratio were found to precede or to coincide with an increase in the T2-weighted lesion volume in 5 of 6 patients (with the exception of patient 2).

B₁ receptor expression in samples collected from healthy donors were also analyzed. B₁–actin levels were lower in healthy controls compared with those of patients with MS (in Figure 1 and Figure 2). Kinin B₁–actin levels in healthy controls (mean ± SE, 0.006 ± 0.0004; range, 0.001-0.022) were significantly lower (P < .001, t test) compared with those obtained from patients with MS (mean, 0.124 ± 0.060; range, 0.055-0.216) (data not shown).

COMMENT

We have previously presented data suggesting that B₁ receptor mRNA expression is up-regulated in lymphocytes in patients with MS who have active disease.⁶ We have also demonstrated that B₁ receptor mRNA levels correlate with B₁ protein expression, as measured by Western blot.⁷ This suggests that assessment of B₁ mRNA transcript levels by means of semiquantitative radioactive duplex PCR amplification is a reliable way to monitor B₁ expression on PBMCs.

Using the clinical-MRI-immunological database generated from the previously reported high-frequency MRI study,¹¹,¹² we performed a semiquantitative analysis of B₁ receptor expression on PBMCs collected monthly from 6 patients with MS and compared expression of B₁ receptor transcripts with date-matched clinical, MRI, and immunological data. Unlike our previous study using ex vivo T cells,⁶ cells used for this study had been cryopreserved. Although we could recover sufficient amounts of mRNA from 6 of 10 patients (cutoff at 3 µg per sample) to PCR amplify B₁ receptor and actin transcripts, duplex PCR amplification cycling needed to be increased from 25 to 30 cycles, probably as a consequence of nonspecific RNA degradation caused by long-term storage. The use of cryopreserved samples did not allow adequate protein purification and these samples did not yield enough material to proceed with Western blotting. Furthermore, the current antibodies available to stain for B₁ receptors are not suitable for flow cytometry, limiting our ability to assess B₁ expression at the protein level.

In the present study, we confirm our initial cross-sectional report correlating B₁ receptor expression and clinical disease activity in MS and extend this observation with correlative MRI and immunological parameters. This study demonstrates that changes in B₁ receptor mRNA expression in PBMCs correlate with changes in T2-weighted lesion volume, a well-defined marker of disease activity in MS. We were, however, unable to demonstrate a correlation between B₁ receptor expression and the presence of Gd-enhancing lesions, perhaps as a consequence of the limited number of new Gd-enhancing lesions (n = 15) recorded for these patients during this study. Our study supports the idea that B₁ receptor expression by leukocytes in MS is not a marker of blood-brain barrier dysfunction (reflected by the absence of correlation with Gd-enhancing lesions) but rather a marker of CNS parenchymal damage (reflected by the positive correlation with T2-weighted lesions). We could also show that expression of B₁ receptor in PBMCs correlates with the presence of leukocyte activation markers (IL-2R, CD26, and MHC class II) on the surface of CD4⁺ lymphocytes or on PBMCs. We have previously shown that B₁ receptor is found on leukocyte during neuroinflammation but not in systemic lupus erythematosus or systemic viral illness,⁸ suggesting that B₁ expression may be less responsive than IL-2R and MHC class II molecules to changes in systemic inflammation and, thus, more selective for ongoing events within the CNS.

While absolute levels of B₁ mRNA expression (kinin B₁–actin ratio) were shown to correlate with the EDSS index, changes in the kinin B₁–actin ratio did not. The overall statistical analysis comparing changes in the kinin B₁–actin ratio and changes in the EDSS index was affected by patients 5 and 6 for whom the EDSS values did not correlate with the T2-weighted lesion volume for the
time-frame studied. For those patients B1–actin values correlated with the T2-weighted lesion volume while the EDSS index in those 2 patients remained stable and high (EDSS index, 6). These findings would seem consistent with previous reports that MRI changes correlate with changes in immune responses even when they are in a clinically silent area.

This study further confirms our initial observation that the levels of B1 receptor mRNA in PBMCs obtained from healthy donors are significantly lower and more stable in time when compared with the levels measured in MS-affected derived samples. Our observations from the control group also suggest that B1 receptor mRNA expression by lymphocytes is not influenced by most of the common viral upper respiratory tract infections. The prevalence of respiratory tract infection was equally distributed between the 2 groups. In our previous cross-sectional study8 we found that lymphocytes obtained from patients with non-CNS-directed inflammatory disease (systemic lupus erythematosus) and from patients with noninflammatory CNS disease (epilepsy) do not express B1 receptor mRNA. Only patients with inflammatory polyneuritis showed minimal B1 receptor expression on their lymphocytes. Such patient groups were unavailable for the frequent clinical-laboratory screening studies conducted on the patients with MS. How-

Figure 1. Time course of expression of kinin B1 receptor messenger RNA by peripheral blood mononuclear cells and Expanded Disease Status Scale (EDSS) indexes derived from 6 patients with multiple sclerosis enrolled in a longitudinal correlative study. Kinin B1 receptor–actin ratios were obtained by radioactive-duplex reverse transcriptase–polymerase chain reaction amplification from cryopreserved samples with coded labels. Each graph represents the time-course expression of kinin B1 receptor and EDSS score for individual patients at time points for which a blood sample was collected. Statistical comparison and correlation study are given in the Table.
ever, we do not consider B1 receptor expression on immune cells to be specific for MS, but rather feel it reflects the general state of immune activation that characterizes MS. Our study provides evidence suggesting that B1 receptor expression on PBMNCs correlates with clinical, MRI, and immune parameters in MS and can be used as a marker of disease activity.

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Correspondence: Alexandre Prat, MD, PhD, FRCPc, Neuroimmunology Laboratory and Multiple Sclerosis Clinic, Centre Hospitalier de l’Université de Montréal–Hôpital Notre-Dame, 1560 Sherbrooke E, Montréal, Quebec, Canada H2L 4M1 (a.prat@umontreal.ca).

Author Contributions: Study concept and design: Prat, Biernacki, Orav, Guttmann, Khoury, and Antel. Acquisition of data: Prat, Biernacki, Saroli, Orav, Weiner, and Khoury. Analysis and interpretation of data: Prat, Biernacki, Orav, Khoury, and Antel. Drafting of the manuscript: Prat, Biernacki, Saroli, and Antel. Critical revision of the manuscript for important intellectual content: Prat, Orav, Guttmann, and Khoury. Statistical analysis: Orav and Guttmann. Obtained funding: Antel. Administrative, technical, and material support: Prat, Biernacki, Saroli, and Weiner. Study supervision: Prat.

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Figure 2. Time course of expression of kinin B1 receptor messenger RNA by peripheral blood mononuclear cells and magnetic resonance imaging–derived T2-weighted lesion volumes obtained from 6 patients with multiple sclerosis. Each graph represents the time-course expression of kinin B1 receptor and magnetic resonance imaging T2-weighted lesion volume for individual patients at time points for which the patients underwent magnetic resonance imaging and blood testing. Statistical comparison and correlation study are given in the Table.
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