Induction of Immunologic Tolerance to Myelin Basic Protein Prevents Central Nervous System Autoimmunity and Improves Outcome After Stroke

J. Michael Gee, BS; Angela Kalil, BA; Matthew Thullbery, MS; Kyra J. Becker, MD

Background and Purpose—Animals subjected to an inflammatory insult at the time of stroke are predisposed to the development of an inflammatory autoimmune response to brain. This response is associated with worse neurological outcome. Because induction of immunologic tolerance to brain antigens before stroke onset is associated with improved outcome, we sought to determine whether this paradigm could prevent the deleterious autoimmune response to brain provoked by an inflammatory stimulus at the time of ischemia.

Methods—Male Lewis rats were tolerized to myelin basic protein (MBP) or ovalbumin by intranasal administration before middle cerebral artery occlusion. At the time of reperfusion, all animals received lipopolysaccharide (1 mg/kg intraperitoneal). Behavioral tests were performed at set time intervals.

Results—One month after middle cerebral artery occlusion, lymphocytes from the spleens of MBP-tolerized animals were less likely to evidence an autoimmune response and more likely to evidence a regulatory response (TREG) toward MBP than those from ovalbumin-tolerized animals. Animals that had an inflammatory response toward MBP (a TH1 response) performed worse on behavioral tests than those that did not. Fractalkine, a surrogate marker of inflammation, was elevated in animals with a TH1 response to MBP.

Conclusions—These data extend our previous findings and suggest that deleterious autoimmunity to brain antigens can be prevented by prophylactically inducing regulatory T-cell responses to those antigens.

Key Words: autoimmunity ■ mucosal tolerance ■ myelin basic protein ■ stroke ■ Th1 ■ TREG

Recent data suggest that there is a systemic immunodepression after stroke.1 Teleologically, this immunodepression may be important for preventing autoimmune responses to brain given that the blood–brain barrier is compromised after stroke,2 which allows cells of the immune system to encounter novel brain antigens in the brain3,4 and the periphery.5–11 Another consequence of this immunodepression is enhanced susceptibility to infection,1,12 and studies show that patients in whom an infection develops in the immediate poststroke period have worse outcomes.13–15 Consistent with the premise of stroke-induced immunodepression, we previously showed that animals tend to evidence a regulatory immune response toward brain antigens after experimental ischemia and that development of a detrimental autoimmune response to brain is rare.16 When subjected to a systemic inflammatory insult with lipopolysaccharide (LPS) to mimic poststroke infection, however, a deleterious autoimmune response occurred in the majority of animals.16 This finding may help to explain why patients in whom an infection develops after stroke have worse outcomes.

Whereas an abundance of evidence suggests that inflammation contributes to postischemic brain injury, there are also data showing that induction of a regulatory immune response to brain antigens by mucosal administration of antigen before stroke can improve outcome.17–21 In the present study, we sought to determine whether induction of a regulatory immune response to brain antigen myelin basic protein (MBP) before stroke onset could prevent the deleterious autoimmune response to MBP that follows a systemic inflammatory insult. We also assessed serological markers of the immune response, including antibodies to MBP and systemic levels of fractalkine, because the amount of this chemokine in circulation appears to correlate with disease activity in patients with autoimmune disorders.22–24

Materials and Methods

Animals

Experiments were approved by the Institution’s Animal Care and Use Committee. Male Lewis rats (250 to 300 grams; Charles River) were used for all studies. Rats were handled before tests/surgical procedures and housed 3 per cage to eliminate differences in socialization.

Tolerization Schedule

The experimental paradigm is outlined in Figure 1. Before sham surgery or stroke, either bovine MBP (100 μg/40 μL) or ovalbumin (OVA; 100 μg/40 μL) was instilled into each nostril every other day for a total of 5 doses; surgery was performed 1 to 2 days after the last
instillation. Bovine MBP was chosen for the induction of tolerance because it can prevent experimental allergic encephalomyelitis\textsuperscript{25,26} and was previously shown to improve outcome in experimental models of stroke.\textsuperscript{20,21}

**Middle Cerebral Artery Occlusion**

Anesthesia was induced with 5\% and maintained with 1.5\% halothane. After midline neck incision, the right common carotid, internal carotid, and pterygopalatine arteries were ligated. A monofilament suture (4.0) was inserted into the common carotid artery and advanced into the internal carotid artery.\textsuperscript{27} 38 OVA-tolerized and 28 MBP-tolerized animals were subjected to middle cerebral artery occlusion (MCAO). Animals were maintained at normothermia during surgery. Reperfusion was performed 3 hours after MCAO. In sham-operated animals (27 OVA-tolerized and 15 MBP-tolerized), the suture was inserted into the carotid but not advanced. Rectal temperature and body weight were assessed at set time intervals (Figure 1). Animals were euthanized 1 month after MCAO or sham surgery. All animals received LPS (serotype 026:B6; 1 mg/kg intraperitoneally) 3 hours after MCAO (at reperfusion) or sham surgery.

**Neurological Outcome**

Neurological status was assessed at set time points after MCAO; tests included a modification of the Bederson scale and an adaptation of the "sticky tape test."\textsuperscript{28,29} Sensorimotor performance was assessed using a rotating drum or "rotarod." Average time to fall (100 sec maximum) from a rod rotating at 5 rpm for 3 trials were recorded.

**ELISPOT Assay**

Animals were euthanized 1 month after MCAO/sham surgery and mononuclear cells were isolated from the brain and spleen using previously described methods.\textsuperscript{16,20} Mononuclear cells were cultured (1×10\(^6\) cells/well) for 48 hours in 96-well plates (MultiScreen-IP; Millipore) in media alone or in media supplemented with MBP (25 \(\mu\)g/mL), OVA (25 \(\mu\)g/mL), or LPS (5 \(\mu\)g/mL). Lymphocyte reaction to whole MBP was used in these assays because the relevant epitope of MBP in this experimental scenario is unknown and unlikely to be that associated with encephalomyelitis. Furthermore, MBP is highly conserved among mammals and an autoimmune response to bovine MBP was previously shown to be associated with worse outcome in experimental stroke.\textsuperscript{16,50} All experiments were performed in triplicate. Antigen-specific secretion of interferon (IFN) \(\gamma\) (in comparison to unstimulated cells) was used as an indicator of the Th1 response; antigen-specific secretion of transforming growth factor (TGF) \(\beta\) was used as an indicator of the Th2 response. Spots were counted under a dissecting microscope by 2 independent investigators blinded to treatment status. Results are expressed as the ratio of the increase in MBP-specific IFN-\(\gamma\)-secreting cells to the ratio of the increase in MBP-specific TGF-\(\beta\)-secreting cells. Based on previous ELISPOT data from our laboratory, the lower interquartile range for this ratio in animals sensitized to MBP (by injection in complete Freund’s adjuvant) was 1.48. We thus considered having a Th1 response to MBP if the ratio of the IFN:TGF response was ≥1.48; conversely, a Treg was considered to be induced if this ratio was ≤0.68 (ie, 1.00/1.48).

Among animals undergoing MCAO, ELISPOT assay was performed on the brains of 17 OVA-tolerized and 16 MBP-tolerized animals. Among sham-operated animals, ELISPOT assay was performed on the brains of 16 OVA-tolerized and 15 MBP-tolerized animals. In some animals the brains were frozen at euthanization but ELISPOT analyses were still performed on spleen.

**Enzyme-Linked Immunosorbent Assays**

Blood was collected by cardiac puncture at the time of euthanization. Serum was stored at -80\°C until use. Fractalkine levels were assessed using a commercially available kit (R&D Systems). Titers of IgG antibodies specific for MBP were measured using indirect enzyme-linked immunosorbent assay. Data are presented as relative absorption units.

**Statistics**

Categorical data were evaluated using the \(\chi^2\) test statistic. Parametric data were compared using the \(t\) test, paired \(t\) test, or ANOVA, and expressed as mean±SEM. Nonparametric data were compared using the Mann-Whitney \(U\) test. Significance was set at \(P\leq0.05\).

**Results**

**OVA-Tolerized Versus MBP-Tolerized Animals**

Overall mortality after MCAO and LPS administration was 34.2\% (13/38) in OVA-tolerized and 17.9\% (5/28) in MBP-tolerized rats (not significant). Mortality in sham-operated
animals that received LPS was 1 of 15 (6.7%) in MBP-tolerized animals and 0 of 27 OVA-tolerized animals (not significant).

Temperatures were similar in OVA-tolerized and MBP-tolerized animals at all time points after MCAO and sham surgery (data not shown).

Animals treated with MBP regained more weight over the course of the month after MCAO than animals treated with OVA (Figure 2a). Neurological scores tended to be lower (better) (supplemental Figure I, available online at http://stroke.ahajournals.org) and the time to notice adhesive tape on the paw was shorter in MBP-tolerized animals. MBP-tolerized animals also spent longer times on the rotarod before falling (Figure 2b). Sham-operated animals performed consistently better on all tests and there were no differences in the neurological performance of OVA-treated and MBP-treated sham-operated rats (data not shown).

**Difference Between the Immune Response in Brain and Spleen**

A differential was seen in the ability of lymphocytes from brain and spleen to respond to mitogenic stimuli (ie, LPS) in vitro. Among a group of naive unmanipulated animals \( n = 5 \), fewer cells from brain than spleen responded to LPS by the secreting IFN-\( \gamma \) \((15.7 \pm 3.7 \text{ per } 1 \times 10^5 \text{ vs } 129.3 \pm 17.6 \text{ per } 1 \times 10^5; P=0.004)\). To account for differences in spontaneous secretion of IFN-\( \gamma \) by brain-derived lymphocytes and by splenocytes, the relative increase in the number of IFN-\( \gamma \)-secreting cells after LPS stimulation was assessed. Again, brain-derived lymphocytes seemed to respond less well than splenocytes to this mitogen \((118\% \pm 20\% \text{ vs } 177\% \pm 22\%; \ P=0.056)\). After sham-surgery, there was similar a differential in the percentage of brain-derived lymphocytes and splenocytes responding to LPS \((86\% \pm 9\% \text{ vs } 131\% \pm 17\%; \ P=0.040)\). After MCAO the response to LPS in spleen is enhanced. The difference in the percentage of cells from brain and spleen responding to LPS is thus more marked \((101\% \pm 6\% \text{ vs } 214\% \pm 32\%; \ P=0.001; \text{ Figure 3})\).

![Figure 2. Effect of mucosal administration of OVA and MBP on outcome. Animals tolerized to MBP gained more weight over the month after MCAO than animals tolerized to OVA (a). MBP-treated animals performed better on the rotarod than OVA-tolerized animals (b). *\( P \leq 0.05 \), **\( P \leq 0.01 \).](http://stroke.ahajournals.org/figCanvas/10.1161/STROKEAHA.110.511005)
Animals demonstrating a Th1* response to MBP in brain had higher body temperatures 72 hours after MCAO than animals without a Th1* response to MBP (37.8±0.1°C vs 37.0±0.1°C; *P*=0.009); animals with a Th1* response to MBP in brain also regained weight more slowly than animals without (Figure 5a). Development of a TREG response did not influence weight gain (Figure 5b). Neurological scores after MCAO were similar in animals with and without a Th1* response to MBP in brain (supplemental Figure I). Animals with a Th1* response to MBP in brain, however, performed consistently worse on the rotarod (shorter times to fall) after MCAO (Figure 5c), whereas animals with a TREG response performed better on this task (Figure 5d). Furthermore, the degree of the immune response to MBP in brain correlated with rotarod performance: the more robust the response to MBP, the worse the performance (*P*=0.020). The effect of a Th1* response to MBP in spleen was not predictive of outcome (supplemental Figure II).

Table. Immunologic Response to MBP in Brain Lymphocytes and Splenocytes 1 Month After MCAO

<table>
<thead>
<tr>
<th></th>
<th>OVA (N=17)</th>
<th>MBP (N=16)</th>
<th>P</th>
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<tbody>
<tr>
<td>Brain-Derived Lymphocytes</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No response</td>
<td>6 (35%)</td>
<td>10 (62%)</td>
<td>0.084</td>
</tr>
<tr>
<td>Th1*†</td>
<td>6 (35%)</td>
<td>1 (6%)</td>
<td></td>
</tr>
<tr>
<td>Treg†</td>
<td>5 (29%)</td>
<td>5 (31%)</td>
<td></td>
</tr>
<tr>
<td>Splenocytes</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No response</td>
<td>13 (62%)</td>
<td>10 (62%)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Th1(+)†</td>
<td>8 (38%)</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Treg†</td>
<td>0</td>
<td>6 (38%)</td>
<td></td>
</tr>
</tbody>
</table>

*Animals were considered to have a Th1* response if the ratio of the number of MBP-reactive lymphocytes secreting IFN to those secreting TGF was >1.48.
†Animals were considered to have a Treg response if the ratio of the number of MBP-reactive lymphocytes secreting IFN to those secreting TGF was <0.68.

The number of animals in which the spleen was assessed was greater than the number of animals in which the brain was assessed because the brains of some animals were frozen for immunohistochemistry.
Peripheral Markers of the Immune Response in Ischemic Animals

Fractalkine levels were higher in animals with a Th1 response to MBP in brain than in those with no response to MBP (471.9±96.3 pg/mL vs 274.2±25.4 pg/mL; P=0.019). Fractalkine levels were similar in animals with a Th1 response to MBP in spleen and those without (429.9±83.1 pg/mL vs 306.4±31.5 pg/mL), but among animals with a Th1 response, serum fractalkine levels correlated to the degree of the cellular immune response to MBP (P=0.038).

Discussion

In previous studies we showed that mucosal administration of MBP before MCAO decreased infarct size and was associated with induction of a Treg response to MBP. The primary aim of the present study was to determine if the paradigm of mucosal tolerance could also be used to prevent a Th1 response to MBP in a model of stroke associated with a high prevalence of such an autoimmune response, specifically, MCAO with LPS administration. Mortality was high in OVA-tolerized animals, likely reflecting the severity of the stroke model (3 hours of MCAO and the concomitant dose of LPS). Because ELISPOT data are available only for surviving animals, the animals with the most severe strokes (and likely the most robust inflammatory responses) were eliminated from analysis by death. The high mortality in OVA-treated animals thus dilutes the effect of MBP treatment on the immunologic outcome. Nonetheless, we found that pre-ischemic induction of mucosal tolerance to MBP suppresses the Th1 response to MBP and increases the probability of a Th3, or Treg, response to this antigen. The present study also extends our previous findings that demonstrated that the paradigm of mucosal tolerance improves early neurological outcome after stroke by now showing that this improvement is sustained over the period of a month. Given that the neurological scores of OVA-tolerized and MBP-tolerized animals were similar immediately after MCAO, the difference in neurological outcome is likely attributable to difference in the immunologic status of these animals. The possibility that the pre-ischemic induction of a Treg response to MBP led to smaller infarcts, which in turn reduced the chances of a Th1 response to MBP must also be considered.
The potential benefit to postischemic induction of a Treg response to MBP therefore needs to be addressed.

That the relationship between neurological outcome and the immunologic response in brain was more robust than in spleen is not surprising considering studies in animals with experimental allergic encephalomyelitis (EAE). In EAE, MBP reactive T cells are found in the central nervous system (CNS) of animals with clinical disease but are absent from the CNS during periods of recovery.\textsuperscript{13} Our findings similarly suggest that there is selective retention of MBP-reactive T cells in the brains of animals with active CNS inflammation leading to worse behavioral outcomes in these animals. Unfortunately, the procedures for isolating lymphocytes from brain for the ELISPOT assay do not allow for simultaneous histological analyses. The presence of a Th1 response to MBP in spleen but not in brain in some animals suggests that an autoimmune response to brain occurred but that the autoreactive cells are not pathological in their present state of activation; however, it is possible that reactivation of these cells, either by another inflammatory stimulus or by ischemic insult, could lead to inflammation within the CNS.

A difference in the ability of lymphocytes isolated from the brain and spleen to respond to mitogenic, and presumably antigenic, stimuli in vitro was detected in this study. It is now widely appreciated that the supposed “immune privilege” of the brain is only relative and that the response of immune cells is regulated within this organ by a number of different mechanisms.\textsuperscript{32–36} One consequence of this endogenous immunoregulation is that lymphocytes in brain have a reduced capacity to produce IFN-γ and other proinflammatory cytokines.\textsuperscript{32,34,35} Teleologically, the CNS-mediated immunosuppression serves to prevent the development of CNS autoimmunity. As we have shown in previous studies, however, inflammation induced by systemic administration of LPS in the setting of cerebral ischemia can lead to autoimmune responses to brain; furthermore, animals that develop a Th1 response to brain (as opposed to spleen). Furthermore, there was a relationship between the robustness of the response to MBP after MCAO. This relationship is most apparent in animals evidencing a Th1 response to MBP in brain (as opposed to spleen). Furthermore, there was a relationship between the robustness of the response to MBP and performance on the rotarod, suggesting that the autoimmune response to brain is more than an epiphenomenon. In addition, we show that animals have a humoral immune response to MBP after stroke and the titers of anti-MBP antibodies correlate with the degree of the systemic cellular immune response to this antigen. Clinical studies demonstrate the development of antibodies to brain in patients after stroke, but the pathological consequences of this humoral response are not known.\textsuperscript{37–39}

Fractalkine (CX3CL1) is a membrane-bound chemokine that is constitutively expressed on the surface of neurons; its expression can also be induced on astrocytes and endothelium.\textsuperscript{40,41} Because of its unique structure, fractalkine can be cleaved from the cell surface and released into the circulation;\textsuperscript{42} excitotoxins are just one of the many different stimuli that lead to cleavage of fractalkine from the neuronal surface.\textsuperscript{43} Cells expressing receptors for fractalkine (CX3CR1) include monocytes, lymphocytes, and microglia, and fractalkine is a potent chemoattractant for these cells. Based on these properties of fractalkine, a role for the chemokine has been suggested in a number of neuroinflammatory disorders. Because fractalkine is upregulated in neurons and endothelial cells within the ischemic penumbra after experimental stroke, it could potentially contribute to the postischemic inflammatory response.\textsuperscript{40} In fact, animals deficient in fractalkine experience better outcomes after cerebral ischemia/reperfusion, although the mechanisms by which the absence of fractalkine provides benefit is unknown.\textsuperscript{44}

Our data show that circulating levels of fractalkine are higher in animals with a Th1 response to MBP in brain, which would be consistent with increased CNS expression (and cleavage) of this chemokine in brain. The fact that Th1 cells preferentially express the fractalkine receptor, and thereby preferentially respond to fractalkine, serves to polarize and augment the immune response.\textsuperscript{45} In patients with autoimmune disorders, serum levels of fractalkine correlate with disease activity.\textsuperscript{22–24} Given that the cellular immune response to MBP in spleen and circulating fractalkine levels were correlated in our study, it suggests that this chemokine could also be used as a marker of ongoing inflammation in the brain. Further studies to compare the histological expression of fractalkine and the cellular immune response in brain (ie, influx of lymphocytes) with circulating fractalkine levels and the Th1 response to brain are warranted.

Because infection is common after stroke and associated with worse outcome, our model of poststroke inflammation (MCAO+LPS) is clinically relevant. LPS, a component of the Gram-negative bacterial cell wall, is a potent inflammatory stimulus that initiates the innate immune response through stimulation of Toll-like receptor-4.\textsuperscript{46} We used LPS to initiate the innate immune response because most poststroke infections are caused by Gram-negative organisms.\textsuperscript{15} The ability of other inflammatory stimuli (which initiate inflammation through activation of other Toll-like receptors) to precipitate an autoimmune response after stroke needs to be investigated.

In these experiments we focused on the immune response to MBP, both as potentially damaging (Th1) and beneficial (Treg). It is possible that similar immunologic responses occur to other CNS antigens. Despite the fact that regulatory T cells are activated in an antigen-specific fashion, they secrete cytokines that influence the immune response in an antigen nonspecific fashion.\textsuperscript{47} This fact leads to the phenomenon known as “bystander suppression” and allows for induction of a therapeutic immunomodulatory response even when the pathogenic antigen leading to a harmful autoimmune response is unknown.\textsuperscript{48,49} Our findings may explain why infection is associated with worse outcome from stroke and suggest a possible therapeutic approach to limit the immunologic consequences of the infection.

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Disclosures
None.

References


Figure 1. Neurological scores after MCAO. There are no significant differences in the neurological scores of OVA and MBP tolerized animals after MCAO (a). There are also no differences in the neurological scores based on the immune response to MBP (Th1+ vs Th1-; (b) or (Th1+ vs TREG vs no response; (c) in brain.
Figure II. T\textsubscript{H}1 response to MBP in spleen and outcome. The immune response to MBP in spleen (T\textsubscript{H}1\textsuperscript{+} vs T\textsubscript{H}1\textsuperscript{-}) was not indicative of neurological score (a), weight gain (b) or rotarod performance (c) after MCAO.
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