The Peroxynitrite Scavenger Uric Acid Prevents Inflammatory Cell Invasion into the Central Nervous System in Experimental Allergic Encephalomyelitis through Maintenance of Blood-Central Nervous System Barrier Integrity

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Central nervous system lesions containing activated cells of the monocyte lineage and nitrotyrosine residues, evidence of the local formation of peroxynitrite (ONOO\(^{-}\)), are pathological hallmarks of multiple sclerosis (MS) and experimental allergic encephalomyelitis (EAE) in conventional mouse models (1–5). In both cases, inflammatory environments producing NO\(^{-}\), NO\(^{2}\), and superoxide. Unlike NO\(^{-}\), ONOO\(^{-}\) is highly toxic and capable of causing pathogenic changes through a variety of mechanisms (13, 14). Thus, although the etiologies of MS and EAE may differ, the latter provides models in which potential therapeutic strategies against shared pathological attributes can be studied. We have previously demonstrated that the development of clinical signs of EAE in SWX/J14 mice immunized with proteolipid protein peptide (PLP\(_{139-151}\)), as well as PLSIL/F\(_{1}\) and IFN-\(\gamma\) receptor knockout mice immunized with myelin basic protein (MBP) can be curtailed by the aggressive administration of uric acid (UA) (1, 15), a known ONOO\(^{-}\) scavenger (16, 17). UA treatment also promotes the recovery of mice with EAE from clinical signs of the disease (15). In this case, nitrotyrosine, a marker of ONOO\(^{-}\) reactivity, disappears from foci of inflammation in the CNS within 4 days of UA treatment (5). Therefore, although it is reasonable to speculate that the protective effect of UA in EAE is through the inactivation of ONOO\(^{-}\), or a closely related molecule, produced at the site of damage in the CNS, there are a number of observations that confound this interpretation. First, unlike humans, in which UA is the end product of purine metabolism, mice rapidly metabolize UA further to allantoin, which does not interact with ONOO\(^{-}\) (16). In our experiments, after a single 10-mg i.p. dose, elevated UA levels are detectable in sera for \(<2\) h (15). A minimum of four daily doses is required to prevent severe EAE in MBP-immunized PLSIL mice, meaning that UA is available for entry into the CNS from serum for \(<8\) h in a 24-h period. Second, the blood-CNS barrier in healthy animals is essentially impervious to UA and, therefore, UA does not have access to the sites where lesions develop until the blood-CNS barrier becomes compromised as a result of the disease process (18, 19). Finally, four daily doses of UA begun 5–10 days before the appearance of clinical signs of EAE in control mice, often prevents both the development of the disease as well as the associated CNS inflammatory response (5, 15). Despite the association between high UA levels and gout in humans, is it possible that UA has anti-inflammatory properties? Could the administration of a large quantity of particulate UA to the peritoneum have some deleterious effect on the development of the immune response to MBP in vivo, as measured by the production of MBP-specific Ab and the induction of MBP-specific T cells. The appearance of cells expressing mRNA for inducible NO synthase in the circulation of MBP-immunized mice was also unaffected by UA treatment. However, in UA-treated animals, the blood-CNS barrier breakdown normally associated with EAE did not occur, and inducible NO synthase-positive cells most often failed to reach CNS tissue. These findings are consistent with the notion that UA is therapeutic in EAE by inactivating ONOO\(^{-}\), or a related molecule, which is produced by activated monocytes and contributes to both enhanced blood-CNS barrier permeability as well as CNS tissue pathology. The Journal of Immunology, 2000, 165: 6511–6518.

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4 Abbreviations used in this paper: ONOO\(^{-}\), peroxynitrite; MS, multiple sclerosis; EAE, experimental allergic encephalomyelitis; UA, uric acid; MBP, myelin basic protein; iNOS, inducible NO synthase; PC, peritoneal cells; DHR123, dihydrorhodamine 123; SIN-1, 1-morpholinosydnonimine hydrochloride.

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response to myelin Ags that is unrelated to its capacity to inactivate ONOO-? To answer these questions and provide further insight into the protective role of UA in CNS inflammatory disease we have determined the effects of UA treatment on the development of MBP-specific immunity, blood-CNS barrier permeability, and CNS inflammation.

Materials and Methods

Measurement of ONOO–-mediated oxidation

Potential ONOO– scavengers and UA derivatives (all purchased from Sigma, St. Louis, MO, except for 1,3,7,9-tetramethylyllic, which was obtained from ICN Biomedicals, Costa Mesa, CA and 2-(mercaptoethyl)-guanidine, which was the gift of Dr. C. Szabo, Inotek, Beverly, MA) were titrated in the presence of 5 μM dihydrodihydrate 123 (DHR123; Molecular Probes, Eugene, OR) and 100 μM 3-morpholinosydnonimine hydrochloride (SIN-1; Alexio Biosciences, San Diego, CA) or in the presence of 5 μM DHR123 and 104 activated cells of the mouse monocyte-macrophage cell line RAW. RAW 264.7 cells (American Type Culture Collection, Manassas, VA) were grown to 80% confluence, activated with 1 μg/ml LPS (Escherichia coli serotype 055:B5; Sigma). ONOO–-mediated oxidation was measured in vitro by the conversion of DHR123 to fluorescein. Fluorescence was measured in a multimode fluorometer (Cytofluor II; Molecular Probes, Eugene, OR) supplemented with 4 μM t-glutamine (Life Technologies, Inc., Burlington, MA) with an excitation wavelength of 485 nm and an emission wavelength of 530 nm.

Western analysis of ONOO–-mediated tyrosine nitration

BSA (1 mg/ml) and FBS (1:20 in PBS) were incubated with 1 mM SIN-1 for 2 h at 37°C in the presence or absence of NaHCO3 (10 mM) and UA (200 μM). Immediately after incubation, 5 μl of each sample was separated on a 10% SDS-polyacrylamide gel and transferred onto a polyvinylidene difluoride membrane (NEN, Boston, MA) (22). Nitrotyrosine-containing proteins were detected with rabbit polyclonal anti-nitrotyrosine Ab (Upstate Biotechnology, Lake Placid, NY) and developed with a diamino benzidine substrate using the Vectastain detection kit according to the manufacturer’s recommendations (cat. no. PK-6101; Vector Laboratories, Burlingame, CA).

Immunization and treatment of mice

EAE was induced in 8- to 10-wk-old female PLSJL mice (the Jackson Laboratory, Bar Harbor, ME) by s.c. immunization with 100 μg MBP in CFA supplemented with 4 mg/ml Mycobacterium tuberculosis H37RA (Difco, Detroit, MI) on day 0, followed by i.p. injection of 400 ng pertussis toxin (List Biological Laboratories, Campbell, CA) on days 0 and 2. MBP was prepared in the laboratory as previously described (23). In this model, EAE can range in onset and severity with 60–100% of the mice developing clinical signs of the disease between 15 and 30 days after immunization. EAE can range in onset and severity with 60–100% of the mice developing clinical signs of the disease between 15 and 30 days after immunization.

In vitro assays of immune function

Single cell suspensions were prepared from the spleens and inguinal and axillary lymph nodes of 5–10 MBP-immunized PLSJL mice by teasing through stainless steel wire mesh in PBS. Red cells were lysed by hypotonic shock. After a minimum of two washes by centrifugation in PBS, cells were resuspended in MEM α modification (Life Technologies, Grand Island, NY) supplemented with 4 μM t-glutamine (Life Technologies, Inc., Burlington, MA) with an excitation wavelength of 485 nm and an emission wavelength of 530 nm.

Analysis of MBP-specific Abs in ELISA

Ab specificity and isotype were assessed in solid phase ELISA. Plates (Polysorb; Nalge Nunc) were coated at room temperature with 2 μg/ml MBP diluted in PBS and incubated overnight in a humidified chamber. The plates were washed with PBS containing 0.05% Tween 20 and blocked with 1% BSA in PBS for 1 h before the addition of serum samples. Samples were diluted 1:100 in PBS and titrated 2-fold down the plate. Following a 2-h incubation at room temperature, plates were washed with PBS containing 0.05% Tween 20 to remove any unbound primary Ab. Bound Ab was detected by the addition of alkaline phosphatase-conjugated rat monoclonal anti-mouse IgG1 (1:2000 in PBS; Pharmingen, San Diego, CA) using p-nitrophenyl phosphate (Sigma) in 0.1 M glycine buffer as a substrate. Absorbance was read in a microplate spectrophotometer (Bio-Tek, Winooski, VT) at 405 nm.

Molecular analysis of iNOS mRNA expression

Total mRNA was extracted using TRIzol (Life Technologies) from lymph node, spinal cord, and PBL isolated from blood by centrifugation on Lymphoprep-M (Cedarlane Laboratories, Hornby, Ontario, Canada). RT-PCR detection of iNOS mRNA was performed using the Access RT-PCR System (Promega, Madison, WI) according to the manufacturer’s instructions. RNA was transcribed and amplified, as previously described (24), using mouse iNOS primers: sense TTC CAG AGT TTC TGG CAG CA; antisense TCT TTA CTC AGT GCC AGA AG, which yield a 524-bp product. RT-PCR products were separated on a 1.5% agarose gel containing ethidium bromide (0.5 μg/ml) and visualized under UV illumination. The 524-bp fragment was excised from the gel and quantitated using a Fluor-S Multi Imager with Quantity One Software (Bio-Rad, Richmond, CA). Specificity of amplification was confirmed by sequencing the PCR products.

Assessment of blood-CNS barrier permeability

Blood-CNS barrier permeability was assessed using a modification of a previously described technique in which sodium fluorescein is used as a tracer molecule (25, 26). Mice received 100 μl of 10% sodium fluorescein in PBS i.v. under isoflurane anesthesia. After 10 min to allow circulation of the sodium fluorescein, cardiac blood was collected and the animals were transcardially perfused with PBS/heparin (1000 U/L). Sodium fluorescein uptake into the spinal cord was measured using a modification of the method of Trout et al. (27). In brief, spinal cord tissue was homogenized in 1.5 ml cold 7.5% TCA and centrifuged for 10 min at 10,000 × g to remove insoluble precipitates. Following the addition of 0.25 ml 5 N NaOH, the fluorescence of a 100-μl supernatant sample was determined using a Cytoflour II fluorometer at excitation 485 nm and emission 530 nm. Serum levels of sodium fluorescein were assessed as described previously (27). Standards (0.125–4 μg/ml) were used to calculate the sodium fluorescein content of the samples in micrograms. Sodium fluorescein uptake into spinal cord tissue is expressed as (μg fluorescence spinal cord/mg protein)/(μg fluorescence serum/μg blood) to normalize values for blood levels of the dye at the time of sacrifice.
Results

Capacity of UA to inhibit ONOO\(^{-}\) reactivity in vitro

Our approach to examining the contribution of ONOO\(^{-}\) to the pathogenesis of EAE has been to determine whether reagents that interfere with its chemical reactions also inhibit the development or progression of the disease (1, 5, 15). Table I summarizes the results of a survey of a variety of compounds that have been assessed for the capacity to inhibit oxidation of DHR\(_{123}\) by ONOO\(^{-}\) produced either chemically by SIN-1 or biologically by LPS-stimulated RAW monocytes. UA, a purine metabolite and known ONOO\(^{-}\) scavenger (16, 17), proved to be the most effective reagent tested, particularly in the more biologically relevant RAW cell assay. UA was selected for further study because of its activity against ONOO\(^{-}\) and the availability of extensive information concerning its toxicity and metabolism in humans where, unlike mice, it is found at relatively high levels (28).

Because of the association between ONOO\(^{-}\) and tyrosine nitration in EAE (5), we next assessed the ability of UA to interfere with this process in vitro. The effects of UA on tyrosine nitration in the presence and absence of NaHCO\(_3\) were compared, as the nitrating activity of ONOO\(^{-}\) is enhanced in the presence of carbonyl anion, which is found in a biological milieu (29, 30). At levels comparable to those found in the sera of normal humans (5 mg/dl), UA effectively inhibited the SIN-1-mediated nitration of tyrosine residues in BSA and various FBS proteins regardless of whether or not carbonyl anion was present (Fig. 1). Taken in context with our previous finding that UA treatment of mice with EAE reduces tyrosine nitration in the CNS (5), these results are consistent with the hypothesis that UA is therapeutic in EAE through inactivating ONOO\(^{-}\).

Table I. Survey of potential ONOO\(^{-}\) scavengers

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Effective dose(_{50}) ((\mu)M)(^a)</th>
<th>SIN assay</th>
<th>RAW assay</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uric acid</td>
<td>1.0</td>
<td>0.6</td>
<td></td>
</tr>
<tr>
<td>1-Methyluric acid</td>
<td>0.5</td>
<td>13</td>
<td></td>
</tr>
<tr>
<td>3-Methyluric acid</td>
<td>2.3</td>
<td>18</td>
<td></td>
</tr>
<tr>
<td>9-Methyluric acid</td>
<td>1.0</td>
<td>nd</td>
<td></td>
</tr>
<tr>
<td>1,3-Dimethyluric acid</td>
<td>1.5</td>
<td>34</td>
<td></td>
</tr>
<tr>
<td>1,7-Dimethyluric acid</td>
<td>2.8</td>
<td>17</td>
<td></td>
</tr>
<tr>
<td>3,7-Dimethyluric acid</td>
<td>70</td>
<td>43</td>
<td></td>
</tr>
<tr>
<td>1,3,7,9-Tetramethyluric acid</td>
<td>&gt;3000</td>
<td>&gt;3000</td>
<td>&gt;3000</td>
</tr>
<tr>
<td>Xanthine</td>
<td>&gt;3000</td>
<td>&gt;3000</td>
<td>&gt;3000</td>
</tr>
<tr>
<td>Allantoin</td>
<td>&gt;3000</td>
<td>&gt;3000</td>
<td>&gt;3000</td>
</tr>
<tr>
<td>(2-Mercaptoethyl)-guanidine</td>
<td>6.0</td>
<td>750</td>
<td></td>
</tr>
<tr>
<td>Ascorbic acid</td>
<td>2.5</td>
<td>15</td>
<td></td>
</tr>
<tr>
<td>1-Cysteine</td>
<td>18</td>
<td>&gt;3000</td>
<td></td>
</tr>
<tr>
<td>1-Cysteine methyl ester</td>
<td>9.0</td>
<td>&gt;3000</td>
<td></td>
</tr>
<tr>
<td>2-Mercaptoethylamine-HCl</td>
<td>19</td>
<td>&gt;3000</td>
<td></td>
</tr>
<tr>
<td>Glutathione reduced</td>
<td>78</td>
<td>&gt;3000</td>
<td></td>
</tr>
<tr>
<td>l(+)-penicillamine</td>
<td>7.0</td>
<td>&gt;3000</td>
<td></td>
</tr>
<tr>
<td>N-acetyl-L-cysteine</td>
<td>78</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>3-[3-cholamidopropyl]-</td>
<td>&gt;5000</td>
<td>&gt;3000</td>
<td></td>
</tr>
<tr>
<td>dimethylammonio]-2-hydroxy</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1-propanesulfonate</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DMSO</td>
<td>ND</td>
<td>&gt;3000</td>
<td></td>
</tr>
</tbody>
</table>

\(^a\) Activated RAW cells were cultured with various ONOO\(^{-}\) scavengers in the presence of DHR\(_{123}\) and SIN-1 as detailed in Materials and Methods. After 1-h incubation, rhodamine fluorescence was measured as an indicator of ONOO\(^{-}\) production.

\(^b\) The effective dose\(_{50}\) is calculated as the dose required to reduce conversion of DHR\(_{123}\) to rhodamine\(_{123}\) by 50%, using 100 \(\mu\)M SIN-1 or 250,000 RAW cells per culture stimulated with LPS to generate ONOO\(^{-}\).

Effects of UA on immune cell function in vitro

Although the presence of relatively high levels of UA in humans makes it highly unlikely that UA has deleterious effects on human lymphocytes or APC, it is within the realm of possibility that UA is therapeutic in EAE through a direct inhibitory effect on the activity of these cells in mice. A likely target of any untoward effect of exogenous UA administration may be peritoneal macrophages as i.p. injection of a 10-mg suspension of UA was required to sufficiently raise serum UA levels in mice in the face of rapid metabolism of the molecule (15). We have previously demonstrated that UA does not suppress NO\(^{-}\) production by LPS-activated RAW cells (15). However, as suggested by the experiment summarized in Table II, UA is apparently taken up by peritoneal macrophages following i.p. administration of a 10-mg suspension. Macrophages recovered from the peritoneum of mice treated for 90–120 min with UA inhibit the detection of ONOO\(^{-}\) in the DHR\(_{123}\) assay (Table II). This is presumably due to the release of sequestered UA into the medium as after 60 min of culture only the supernatant had the capacity to inhibit DHR\(_{123}\) oxidation (data not shown). The fact that macrophages obtained 30 min after UA administration do not have this effect indicates that UA uptake may be an active process requiring a greater period of time (Table II). The reduced inhibition of DHR\(_{123}\) oxidation seen with peritoneal macrophages recovered 120 min after UA treatment could be the result of the rapid clearance of UA, which occurs in mice (15), or due to trafficking of the cells to other sites. In either case, macrophages potentially involved in the pathogenesis of EAE appear to retain UA for some time after elevated levels have disappeared from the serum (15).

Regardless of whether macrophages pick up UA, its presence appears to have little effect on Ag-specific immunity. As shown in Table III, UA at levels equivalent to those found in human serum (which average 4–6 mg/dl, depending on age and sex) and slightly higher than those attained in the sera of PLSJL mice by the i.p.
administration of 10 mg UA (peak level 30 min postinjection, 3.0 ± 0.4 mg/dl; mean of six mice ± SEM), does not significantly inhibit MBP-specific T cell proliferation in vitro. Effects due to the addition of the UA precursor xanthine or oxidative product allantoin were also minimal (Table III). These results indicate that both the presentation of MBP and T cell recognition of the Ag in EAE are unlikely to be negatively influenced by UA or allantoin.

Effects of UA on the immune response to MBP

Even if UA does not have a direct inhibitory effect on APC or CD4 T cell function, it is possible that its administration may influence some aspect of immunity in mice. As shown in Fig. 2, there is no statistically significant difference between the MBP-specific in vitro proliferative responses of lymph node cells from mice immunized with MBP and treated with saline, allantoin, or UA for 7 and 10 days following immunization. This implies that UA has no effect on Ag presentation or T cell priming and expansion in vivo. The kinetics and magnitude of the MBP-specific proliferative responses of spleen cells from the MBP-immunized, UA-, allantoin-, and saline-treated animals are also essentially the same (Fig. 3), which implies that any effect of UA on the recirculation of T cells or APC from the lymph nodes draining the site of immunization to the spleen is minimal. UA treatment also had no effect on the development of the Ab response to MBP. MBP-specific IgG1 (Fig. 4), IgG2a, and IgG2b (data not shown) Abs appeared in equivalent levels between days 7 and 10 after immunization in both UA- and control-treated mice.

In addition to MBP-specific lymphocytes, activated monocytes expressing iNOS have been implicated in the development of EAE in conventional models (eg., 3, 5, 8, 11) where disease pathogenesis has been associated with CNS inflammation and nitrotyrosine formation (eg., 3, 5). Therefore, we have assessed the effects of UA treatment on the distribution of iNOS-positive cells in PLSJL mice immunized with MBP. UA treatment has no effect on the expression of iNOS mRNA in the lymph nodes of mice immunized with MBP (Fig. 5). Although cells expressing significant levels of iNOS mRNA are not found in the peripheral blood of the majority of nonimmune PLSJL mice, such cells appear in the circulation of most mice between 12 and 16 days after immunization with MBP regardless of whether they have been treated with UA (Fig. 6). At 20 days postimmunization, clinical signs of EAE and the presence of iNOS-mRNA in spinal cord were assessed in control and UA-treated mice that were positive for iNOS mRNA in peripheral

### Table II. Inhibition of ONOO⁻-mediated oxidation by peritoneal macrophages from UA-treated mice

<table>
<thead>
<tr>
<th>Rhodamine₁₂₅ Fluorescence from SIN-1-Elaborated ONOO⁻</th>
<th>In vivo treatment of macrophages added to culture</th>
<th>UA</th>
</tr>
</thead>
<tbody>
<tr>
<td>No addition</td>
<td>None</td>
<td>30 min</td>
</tr>
<tr>
<td>55,282 ± 660</td>
<td>53,606 ± 1,325</td>
<td>53,943 ± 636</td>
</tr>
</tbody>
</table>

a PLSJL mice were either left untreated or treated with a single 10-mg dose of UA i.p. At the indicated intervals the mice were euthanized and peritoneal cells recovered. The cells were washed three times in PBS, isolated by density gradient centrifugation, and then added to cultures containing DHR₁₂₅ and SIN-1 in PBS. After 1-h incubation at 37°C, rhodamine fluorescence in supernatant from the cultures was measured as described in Materials and Methods. Means ± SD of seven samples are shown. Background fluorescence in the absence of SIN-1 was 1736 ± 151.

b Statistically significant inhibition (p < 0.001, by Student’s t test) was evident when macrophages from mice treated with UA for 90 or 120 min were added to the SIN-1 plus DHR₁₂₅ (by comparison with similar cultures containing macrophages from normal mice).

### Table III. Lack of effect of UA on MBP-specific proliferation in vitro

<table>
<thead>
<tr>
<th>[³H]Thymidine Incorporation (mean cpm ± SD)</th>
<th>Unstimulated cultures</th>
<th>MBP (10 μg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>1,069 ± 91</td>
<td>102,897 ± 4,599</td>
</tr>
<tr>
<td>UA</td>
<td>2,487 ± 283</td>
<td>95,882 ± 1,175</td>
</tr>
<tr>
<td>Allantoin</td>
<td>3,262 ± 171</td>
<td>81,867 ± 2,339</td>
</tr>
<tr>
<td>Xanthine</td>
<td>3,327 ± 105</td>
<td>84,644 ± 1,109</td>
</tr>
</tbody>
</table>

a Lymph node cells from PLSJL mice immunized with MBP in CFA were cultured at 2.5 × 10⁶/ml with an equal concentration of unselected spleen cells as APC in the absence or presence of MBP, UA, allantoin, or saline. Proliferation was estimated by a 4-h pulse with [³H]thymidine as detailed in Materials and Methods.
blood cells 4 days previously (Fig. 7). The development of clinical signs of EAE in MBP-immunized PLSJL mice was associated with the appearance of iNOS mRNA in spinal cord tissue. Both the development of clinical signs and the appearance of iNOS-positive cells in spinal cord tissue were, in most cases, prevented by UA administration (Fig. 7). Note that the rare UA-treated animal that expresses iNOS mRNA in the spinal cord does not necessarily exhibit signs of clinical disease.

**Effect of UA treatment on enhanced blood-CNS barrier permeability in EAE**

As the blood-CNS barrier normally becomes compromised in EAE and UA treatment of PLSJL mice with the disease promotes recovery of blood-CNS barrier integrity (5), we thought that the failure of iNOS-positive cells to reach spinal cord tissue in the UA-treated, MBP-immunized animals may be related to a UA-mediated effect on the blood-CNS barrier. Therefore, we tested whether UA administration beginning before the onset of EAE protects against the blood-CNS barrier permeability changes associated with the disease. Such experiments are made difficult to interpret because of several factors including 1) failure of up to 40% of MBP-immunized PLSJL mice to develop EAE within a reasonable period of time; 2) variability in onset of clinical signs of EAE between individual mice; and 3) absence of disease in the majority of UA-treated mice. Because of the latter, both treated and control mice must be sampled based on the predicted onset of disease in the control group. In the experiment shown in Fig. 8, the permeability of the blood-CNS barrier was assessed 18 days postimmunization with MBP when only 6 of the 40 mice had shown mild signs of clinical EAE (score ≤3). Based on blood-CNS barrier permeability, control- and UA-treated animals each segregate into two statistically different groups ($p < 0.001$ for the control groups, and $p < 0.02$ for the UA-treated groups; Mann-Whitney U test). However, although blood-CNS barrier permeability was clearly elevated in 13 of 27 control-treated mice, only 2 of 13 UA-treated mice showed a similar change. UA treatment

**FIGURE 3.** UA treatment has no effect on MBP-specific T cell priming in spleen. Spleen cells from PLSJL mice immunized 10 days previously with MBP in CFA and treated i.p. four times daily with UA, allantoin, or saline beginning on day 0, were cultured at $1 \times 10^6$ /ml in the presence (closed symbols) or absence (open symbols) of MBP (10 μg/ml). Proliferative response was determined by a 4-h [3H]thymidine pulse. Data points are means ± SD of triplicate samples.

**FIGURE 4.** UA treatment has no effect on the development of an MBP-specific humoral response. Groups of 5–10 PLSJL mice, immunized with MBP in CFA, received four daily i.p doses of 10-mg UA, allantoin, or saline, starting at the time of immunization. The serum Ab response to MBP was assessed in solid phase ELISA with IgG-specific secondary Abs 7 (open symbols, dashed lines) and 10 (filled symbols, solid lines) days after immunization. Normal mouse serum (NMS) was also assayed in the ELISA (closed line). Data is represented as mean ± SD. Serum Ab responses were negligible at 7 days postimmunization and are indistinguishable on the graph.

**FIGURE 5.** iNOS-mRNA expression in the lymph nodes of MBP-immunized mice is not altered by the administration of UA. Groups of 5–10 PLSJL mice were either left naive or immunized with MBP in CFA and treated i.p four times daily with 10-mg UA, allantoin, or saline starting at day 5. Twenty days after immunization, lymph nodes were collected and RNA was isolated and assessed for iNOS-specific mRNA as described in *Materials and Methods*. Symbols represent iNOS mRNA levels of lymph nodes from individual animals.
significantly reduced the incidence of mice with evidence of compromised blood-CNS barriers (χ² test, p < 0.023). A similar relationship was seen in the expression of iNOS mRNA in the spinal cords of control- vs UA-treated mice 20–21 days after MBP immunization (Fig. 9). Control mice segregated into two statistically distinct groups (p < 0.001 by the Mann-Whitney U test) with 12 of 25 mice expressing high levels of iNOS mRNA. Eleven of the 12 mice exhibited clinical scores higher than 2, whereas only one was not sick and another had clinical signs of EAE and mRNA levels within the lower range. In contrast, only one of nine UA-treated animals expressed high levels of iNOS mRNA in the spinal cord. None of these mice showed clinical signs of EAE.

**Discussion**

UA is a highly efficient scavenger of ONOO⁻ whether the latter molecule is generated chemically or biologically. For example, ascorbic acid, another biologically relevant antioxidant, was ~15-fold less effective at inhibiting DHR₁₂₃ oxidation mediated by ONOO⁻ elaborated by activated macrophages. More important in the context of nitrotyrosine formation in MS and EAE (1–5), UA efficiently inhibits ONOO⁻-mediated tyrosine nitration in the presence and absence of a source of carbonyl anion. Apart from inactivating ONOO⁻, UA does not appear to have any significant effect on in vitro measures of immune and inflammatory cell function relevant to EAE. Although peritoneal macrophages appear to take up UA, the expression of iNOS and production of NO⁻ by monocytes in MBP-immunized mice are not diminished by UA treatment. The MBP-specific T cell proliferative response is also unchanged in the presence of UA. Thus it is not surprising that the in vivo immune response to MBP appears to be largely unaffected by the administration of UA even if begun on the day of immunization as opposed to 5–10 days later, which still successfully interferes with the development of clinical EAE (15). The levels of MBP-specific IgG Abs produced by MBP-immunized mice, whether treated with UA or not, are equivalent, as is the capacity...
of T cells recovered from such animals to proliferate in response to MBP in vitro. More importantly, with respect to the putative association between iNOS, ONOO\textsuperscript{-}, and the pathogenesis of EAE, it is readily apparent that UA treatment does not interfere with iNOS expression in the lymph nodes or peripheral blood of mice subjected to the stimulus that triggers EAE. Therefore, we conclude that UA treatment does not affect the MBP-induced activation of monocytes to express iNOS or the circulation of these cells through peripheral blood, a necessary step toward the invasion of CNS tissue. However, UA treatment evidently prevents iNOS-positive cells from entering CNS tissue.

A variety of observations have provided evidence that ONOO\textsuperscript{-} may have deleterious effects on the integrity of the blood-CNS barrier (5, 31–33). The current findings support the hypothesis that one therapeutic mode of action of UA in EAE is through interfering with inflammatory cell invasion into the CNS by blocking ONOO\textsuperscript{-}-mediated permeability changes in the neurovascularus. Presumably, for this to be the case UA would have to be available at the blood-CNS barrier consistently, yet we can only detect it in serum for \(<2\) h following each dose (15). Although it is possible that even the transient presence of UA could sufficiently reduce the effect of ONOO\textsuperscript{-} on the blood-CNS barrier to help maintain its integrity, the fact that peritoneal macrophages are probably taking up UA provides another possible explanation. Peritoneal macrophages from UA-treated mice show peak antioxidant capacity when serum UA levels have already deteriorated. Therefore, monocytes may serve as a short-term reservoir of UA that is not subject to rapid breakdown by urate oxidase in the liver (34) and can be targeted to areas of inflammatory cell accumulation.

The fact that UA treatment is therapeutic against the development of EAE in the rare instance where iNOS mRNA-positive cells appear in the spinal cord despite UA administration is consistent with our previous observation that UA can reverse the progression of clinical disease in mice (15). The mode of action in this scenario is presumably that UA penetrates the compromised blood-CNS barrier and inactivates ONOO\textsuperscript{-} produced at the site of damage. The best evidence supporting this possibility is that UA treatment is associated with inhibition of tyrosine nitration but not iNOS expression in the lesion (5). However, concomitant with recovery from EAE, UA-treated mice also show a return of blood-CNS barrier permeability to normal (5). Our current findings support the concept that a major protective effect of UA in EAE is through the maintenance of blood-CNS barrier integrity, which prevents iNOS-positive cells and, likely, other pathogenic cells and factors from reaching spinal cord tissue. In this case, ONOO\textsuperscript{-}, or a closely related product, must play a principal role in providing activated monocytes access to CNS tissues. Further studies are necessary to determine whether this is through damage or a signaling process and whether there is a contribution from up-regulation of adhesion molecules on vascular endothelial cells in addition to the enhanced physical permeability of the blood-CNS barrier.

We have previously observed significantly lower serum UA levels in a group of MS patients by comparison with age- and sex-matched controls (15). The current findings suggest that UA does not directly inhibit the immune mechanisms responsible for the pathogenesis of EAE, an animal correlate of MS, but instead protects against the development of the disease by preventing blood-CNS barrier breakdown and the associated inflammatory cell invasion into CNS tissues. However, UA treatment also promotes the recovery of mice with pre-existing EAE (5, 15). We theorize that the role of UA in human physiology is to maintain blood-CNS barrier integrity as well as to prevent tissue damage due to ONOO\textsuperscript{-} or associated radicals (35). In this case, low serum UA levels in humans may predispose toward the development of MS and other CNS inflammatory diseases.

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References
10. Bagasra, O., F. H. Michaels, Y. M. Zheng, L. E. Bobrowski, S. V. Spitsin, Z. F. Fu, R. Tawdros, and H. Kropowski. 1995. Activation of the inducible form of nitric oxide synthase in spinal cord tissue of MBP-immunized mice. Three groups of PL/JL mice were immunized with MBP in CFA and treated with 10 mg UA or controls (allantoin and saline) i.p. four times daily beginning on day 5 postimmunization. Twenty days postimmunization, spinal cord samples were collected, and RNA was extracted and assessed for iNOS mRNA expression. As shown, control- and UA-treated mice each segregated into two statistically significant populations (\(p < 0.001\) by the Mann-Whitney U test) based on iNOS mRNA expression in spinal cord tissue. Groups control 1 and UA 1 had iNOS mRNA levels 5-fold greater than the highest level seen in normal mice (117), whereas groups control 2 and UA 2 had average levels less than twice this value. For each group the values obtained fell in the range of the boxes plus bars. The central 50% of the values fell within the boxes, which also contain the median line for the group. One-way ANOVA indicated that the means of the groups (exclusive of UA 1, which only had a single value) are significantly different (\(p < 0.001\)) and Dunnett’s Multiple Comparison post test revealed that only control 1 group differed significantly from normal mice (\(p < 0.01\)).