The Journal of Immunology

The Central Nervous System Inflammatory Response to Neurotropic Virus Infection Is Peroxynitrite Dependent

D. Craig Hooper, Rhonda B. Kean, Gwen S. Scott, Sergei V. Spitsin, Tatiana Mikheeva, Kinjiro Morimoto, Michael Bette, Annette M. Röhrenbeck, Bernhard Dietzschold and Eberhard Weihe

J Immunol 2001; 167:3470-3477; ;
doi: 10.4049/jimmunol.167.6.3470
http://www.jimmunol.org/content/167/6/3470

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

The Central Nervous System Inflammatory Response to Neurotropic Virus Infection Is Peroxynitrite Dependent[1]

D. Craig Hooper,[2]* Rhonda B. Kean,* Gwen S. Scott,* Sergei V. Spitsin,* Tatiana Mikheeva,* Kinjiro Morimoto,* Michael Bette,† Annette M. Röhrenbeck,† Bernhard Dietzschold,* and Eberhard Weihe†

We have recently demonstrated that increased blood-CNS barrier permeability and CNS inflammation in a conventional mouse model of experimental allergic encephalomyelitis are dependent upon the production of peroxynitrite ($ONOO^-$), a product of the free radicals $NO^{\cdot}$ and superoxide ($O_2^{\cdot -}$). To determine whether this is a reflection of the physiological contribution of $ONOO^-$ to an immune response against a neurotropic pathogen, we have assessed the effects on adult rats acutely infected with Borna disease virus (BDV) of administration of uric acid (UA), an inhibitor of select chemical reactions associated with $ONOO^-$. The pathogenesis of acute Borna disease in immunocompetent adult rats results from the immune response to the neurotropic BDV, rather than the direct effects of BDV infection of neurons. An important stage in the BDV-specific neuroimmune response is the invasion of inflammatory cells into the CNS. UA treatment inhibited the onset of clinical disease, and prevented the elevated blood-brain barrier permeability as well as CNS inflammation seen in control-treated BDV-infected rats. The replication and spread of BDV in the CNS were unchanged by the administration of UA, and only minimal effects on the immune response to BDV Ags were observed. These results indicate that the CNS inflammatory response to neurotropic virus infection is likely to be dependent upon the activity of $ONOO^-$ or its products on the blood-brain barrier. *The Journal of Immunology,* **2001, 167: 3470–3477.**

In its natural hosts, sheep and horses, Borna disease virus (BDV),[3] a nonsegmented, negative strand RNA virus, mediates a progressive, fatal neurological disease (reviewed in Ref. 1). A similar outcome is most often the case for BDV infection of rats, the animal model in which much of the study of Borna disease pathogenesis has been performed. Although neurons are the principal cellular targets of BDV infection in the rat, astrocytes and other cell types may become involved as the infection progresses (2–4). Nevertheless, it is the immune response to BDV Ags, rather than BDV infection of neurons and other CNS resident cells, that causes acute lethal Borna disease (reviewed in Ref. 5). The central role of specific immunity in the pathogenesis of acute Borna disease is clearly illustrated by the fact that BDV infection does not cause overt disease in T cell deficient athymic adult rats or in neonatal rats in which immunological tolerance can be readily established (6–8). In both cases, a persistent BDV infection with little evidence of overt disease ensues (6, 7). In the developing rat, BDV infection causes a slow, progressive loss of Purkinje cells, with limited neuropathological and behavioral changes and little evidence of CNS inflammation (9, 10). On the other hand, the acute Borna disease seen in immunocompetent adult rats is associated with extensive CD4 T cell-dependent CNS inflammation and BDV-specific CD8 T cell activity (11–15). Levels of mRNA for IL-1α, IL-2, IL-6, TNF-α, and IFN-γ as well as other immunologically relevant factors all become elevated in the brains of rats acutely infected with BDV (16–19). Both CNS resident cells and invading macrophages may participate in the inflammatory response that contributes to the pathogenesis of acute Borna disease by producing free radicals and other factors that may interfere with the function of, if not kill, neurons.

It is generally accepted that nervous tissue is shielded from the immune system by the blood-brain barrier (BBB), presumably to prevent inflammatory and cytolytic responses from damaging neurons, which have a limited capacity for repair. Restricted expression of class I MHC Ags by neurons (reviewed in Ref. 20) may offer some protection from Ag-specific cytotoxicity, but non-Ag-specific inflammatory mechanisms alone are most likely sufficient to cause substantial CNS damage. Studies of experimental allergic encephalomyelitis (EAE) have provided considerable information concerning CNS inflammatory disease in the absence of an underlying viral infection. In this model, acute neurological disease is triggered by sensitization of CD4 T cells with myelin Ags, and CNS pathology has been associated with free radical production by inflammatory cells (21–23). Peroxynitrite ($ONOO^-$), the product of $NO^{\cdot}$ and $O_2^{\cdot -}$, is believed to be an important toxic molecule in this context (24–27). Whether the reactions attributed to $ONOO^-$ in vivo are in fact mediated by radicals formed by the interaction of $ONOO^-$ and other molecules found in a biological milieu is currently under investigation (28, 29). Therefore, in the context of our work, reference to $ONOO^-$ should be taken as including related intermediates. We have recently provided evidence that, in addition to

*Department of Microbiology and Immunology, Thomas Jefferson University, Philadelphia, PA 19107; and †Department of Molecular Neuroimmunology, Institute of Anatomy and Cell Biology, Philipps University, Marburg, Germany

Received for publication November 14, 2000. Accepted for publication July 9, 2001.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] This work was supported in part by a grant from the Theodore and Vada Stanley Foundation. D.C.H. and S.V.S. were supported in part by a grant to the Biotechnology Foundation Laboratories from the Commonwealth of Pennsylvania and in part by a grant to D.C.H. from the National Multiple Sclerosis Society (RG 2896A8/5). G.S.S. was supported by the Paralyzed Veterans of America Spinal Cord Research Foundation. E.W. and M.B. were supported by the Deutsche Forschungsgemeinschaft (SFB 297 "Mechanisms of neuro-immune-endocrine interactions").

[2] Address correspondence and reprint requests to Dr. D. Craig Hooper, Department of Microbiology and Immunology, Thomas Jefferson University, 1020 Locust Street, JAH Room 454, Philadelphia, PA 19107-6799. E-mail address: douglas.c.hooper@mail.tju.edu

[3] Abbreviations used in this paper: BDV, Borna disease virus; BBB, blood-brain barrier; EAE, experimental allergic encephalomyelitis; iNOS, inducible NO synthase; NT, nitrotyrosine; $ONOO^-$, peroxynitrite; p.i., postinfection; UA, uric acid.

0022-1767/01/$02.00

causing pathological changes in CNS tissues, $ONOO^-$ plays an important role in promoting inflammatory cell invasion into CNS tissues in EAE (30). For instance, administration of uric acid (UA), a natural inhibitor of select chemical reactions associated with $ONOO^-$, to animals with EAE protects the blood-CNS barrier from permeability changes normally associated with the disease (30). As the production of the $ONOO^-$ precursor $NO^\bullet$ is a hallmark of the CNS inflammatory response in both EAE (15, 22) and acute Borna disease (15), we postulate that $ONOO^-$ may have a physiological role in neuroimmune responses to enhance BBB permeability and promote cell invasion into the CNS. To investigate whether this may be the case for a neurotropic virus infection, we have assessed the effects of UA treatment on BBB permeability, CNS inflammation, and clinical disease in immunocompetent adult rats acutely infected with BDV.

Materials and Methods

BDV infection of rats and treatment

Twelve- to 14-wk-old female Lewis rats (Harlan Breeders, Indianapolis, IN) were infected under anesthesia with 30 μl per nostril of a 4% brain homogenate containing 10^6 focus-forming units of BDV. Following infection, animals were examined daily for clinical signs of disease. Scores were assigned on the basis of the presence of the following symptoms: 0, normal; 1, hyperactivity, aggressiveness, extremely excitable, and respond vigorously to loud noises; 2, disturbance of motor functions, ataxia; 3, development of tremors, spasms, convulsions, and paralysis; 4, moribund, no response to stimuli. Beginning at 10–14 days postinfection (p.i.), groups of BDV-infected rats were treated i.p. with two daily doses of 100 mg UA, or its inert oxidation product allantoin, in 1 ml saline or saline vehicle alone at an interval of 8 h. With respect to UA treatment, this dose resulted in an elevation of serum UA levels in the rats from $\sim$0.5 $\pm$ 0.1 SD to a peak of 3.7 $\pm$ 0.7 SD mg/dl in 30 min. Due to the presence of urate oxidase in rats, levels of UA in serum dropped rapidly from 30 min postadministration, becoming half-maximal at $\sim$2 h, but remaining significantly elevated for up to 4 h (1.1 $\pm$ 0.3 at 4 h, $p = 0.01$).

ELISA for BDV-specific Ab

Sera from rats infected with BDV 22–24 days previously and treated with 100 mg UA, allantoin, or saline from day 10 p.i. were assessed for BDV-specific Abs in solid-phase ELISA. Plates (Polysorb; Nalge Nunc International, Rochester, NY) were coated at room temperature with a 2% homogenate of either uninfected or BDV-infected rat brain diluted in PBS and incubated overnight in a humidified chamber. The plates were washed with PBS containing 0.05% Tween 20 (PBS-Tween 20) and blocked with 5% milk in PBS for 1 h before the addition of serum samples. Samples were diluted 1/10 in PBS and titrated 2-fold down the plate. Following a 2-h incubation at room temperature, plates were washed with PBS-Tween 20. Ab was detected using peroxidase-conjugated anti-rat IgG whole molecule (dilution 1/2000; Sigma, St. Louis, MO) with 3,3′,5,5′-tetramethylbenzidine (Sigma) in phosphate-citrate buffer (Sigma) as a substrate. Absorbance was read at 450 nm in a microplate spectrophotometer (Biotek, Winooski, VT).

Real-time quantitative PCR

RNA was isolated from brain tissue of rats infected 22–24 days previously with BDV and treated from day 10 p.i. with two daily i.p. doses of 100 mg UA, allantoin, or with saline vehicle. Rats were anesthetized and perfused with PBS containing heparin (1000 U/L), and then brains were removed and snap frozen. RNA was isolated from brain using TRIzol B (Life Technologies, Grand Island, NY), then DNA contamination was removed by treatment with DNA-free reagent (Ambion, Austin, TX). cDNA was synthesized from 5 μg total RNA using Moloney murine leukemia virus reverse transcriptase (Promega, Madison, WI) and dT_{15} primer. An equivalent of 50 ng total RNA was used for the PCR with Taqman PCR Core Reagent kit (Applied Biosystems, Foster City, CA). Primers and probes were designed using the Web Primer 3 program (http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi). Double-labeled probes were purchased from Integrated DNA Technologies (Coralville, IA), and primers were synthesized by our in-house Nucleic Acid Facility (Kimmel Cancer Institute, Thomas Jefferson University, Philadelphia, PA). The 5′ ends of probes were labeled with reporter dyes Hex or 6-Fam, and 3′ ends with the quencher BHQ-1. Probes and primers were as follows: CD8, probe, TTG CTT GGT AGG GCA GCA TGA CTG GC; 5′ primer, AGC CTT GCA GCC ACC ATC TTT; 3′ primer, TCT CTC CCA TGA GAC GCC AAA. CD4, probe, CCC ACA CAG TGC CTC TGG CCT CCT; 5′ primer, GGC CTG TGG ACC AGA TGA ATG; 3′ primer, GAG AAA CCG TGG CCA GTT GTG. MHC class II, RT1B, probe, TCA GAT GGC CCC TCC AGA CAC CCA; 5′ primer, GGG CAC CAT CTT CAT CAT TCA AG; 3′ primer, TTC TTT CCC AGG GTG TGA CTC AA. GAPDH, probe, AGA AGG TGG TGA AGC AGG CGG C; 5′ primer, TGG AGA AAC CTG CCA AGT ATG; 3′ primer, GTC CTC AGT GTA GCC CAG GAT. Quantitative PCR was performed using a Bio-Rad iCycler iQ Real Time Detection System (Hercules, CA). Data were calculated based on a threshold cycle (Ct) determined as the cycle with a signal higher than that of the background (signal detected in cycles 2–10) plus 10$\times$ its SD. Data are expressed as a fold increase in mRNA expression calculated by exponent (Ct lowest expresser (e.g., normal mice) $-$ Ct test value) divided by the same value determined for the housekeeping gene *GAPDH*.

Immunohistochemistry

Groups of saline- and UA-treated (from day 14 p.i.) rats were anesthetized with ketamine/xylazine 23 days post-BDV infection and transcardially perfused with PBS containing procaine-HCl (5 g/L), followed by Bouin Hollande fixative, as described elsewhere (18). The perfused brains were removed, dissected into anterior, middle, and posterior parts, and postfixed for 24–48 h in Bouin Hollande fixative. After dehydration in a graded series of 2-propanol solutions, tissues were embedded in Paraplast Plus (Merck, Darmstadt, Germany). Deparaffinized serial sections were stained with Giemsa according to standard procedures for the general assessment of histopathology and inflammation, or stained with one of the following primary Abs: the mouse mAb BO18 (dilution 1/1000, a gift from J. Richt, University of Giessen, Giessen, Germany), which recognizes the BDV 38-kDa Ag, a major protein of BDV expressed at all stages of replication; the mouse anti-rat ED1 mAb (dilution 1/200; Camon, Wiesbaden, Germany) as a specific marker for cells of the monocyte/macrophage lineage and activated microglia; the polyclonal rabbit anti-nitrotyrosine (NT) Ab (dilution 1/4000; Chemicon, Temecula, CA), which is a specific marker for highly reactive nitrogen species derived from NO, such as $ONOO^-$, nitrogen dioxide, and nitryl chloride, which lead to the nitration of tyrosine residues in protein chains (31); the polyclonal rabbit anti-inducible NO synthase (iNOS) Ab (dilution 1/400; Santa Cruz Biotechnology, Heidelberg, Germany); the mouse anti-rat CD4 Ab (dilution 1/25; Serotec, Eching, Germany) recognizing rat CD4 cell surface glycoprotein expressed by Th cells; and the mouse anti-rat CD8 Ab (dilution 1/500; Labgen, Frankfurt, Germany) recognizing rat CD8 cell surface glycoprotein expressed by cytotoxic/suppressor T cells. Sections were incubated with the primary Abs overnight at 18°C, followed by an additional incubation for 2 h at 37°C. After several washes in distilled H_2O, followed by rinsing in 50 mM PBS, species-specific biotinylated secondary Abs (dilution 1/200; Dianova, Hamburg, Germany) were applied for 45 min at 37°C. After another series of washes, sections were incubated for 2 h at 37°C with the streptavidin-biotin-HRP complex (Amersham/Buchler, Braunschweig, Germany), as described previously (32), or the ABC reagents (Vectastain; Camon, Wiesbaden, Germany). Immunoreactions were visualized with 3′3-diaminobenzidine (Sigma) enhanced by the addition of 0.08% ammonium nickel sulfate (Fluka, Buchs, Switzerland), resulting in a dark blue staining, as described previously (33). Staining controls consisted of sections treated similarly, but without primary Abs. In these controls, no immunoreactions were visible for any used Ab (data not shown). Sections were analyzed and photographed with an Olympus (Melville, NY) AX 70 light microscope.

BBB permeability

BBB permeability was assessed using a modification of a previously described technique in which fluorescein is used as a tracer molecule (30). Rats, infected with BDV 22–24 days previously and treated with UA, allantoin, or saline from day 14 p.i., received 300 μl of 10% sodium fluorescein in PBS i.v. under isoflurane anesthesia. After 5 min, to allow circulation of the sodium fluorescein, cardiac blood was collected, the animals were transcardially perfused with PBS/heparin, and brains were removed. Brains were weighed, homogenized in 1.5 ml of cold 7.5% trichloroacetic acid, and centrifuged for 10 min at 10,000 $\times$ *g*. Following the addition of 0.25 ml of 5 N NaOH, the fluorescence of 100 μl of supernatant was determined using a Cytofluor II fluorometer (PerSeptive Biosystems, Framingham, MA) with 485 nm excitation and 530 nm emission. Serum levels

of sodium fluorescein were assessed as previously described (30). Standards ranging from 125 to 4000 μg/μl were used to calculate the sodium fluorescein content of the samples in μg. Sodium fluorescein uptake into the brain tissue is expressed as (micrograms fluorescence in brain/milligram protein)/(μg fluorescence in sera/microliter blood) to normalize values for blood levels of the dye at the time of sacrifice.

Results

Effect of UA treatment on acute Borna disease and the BDV-specific immune response

In our model, clinical signs of Borna disease appear in Lewis rats ~20 days after intranasal infection with BDV (17, 18). The disease is rapidly progressive, with the majority of infected rats dying within 3–5 days of the onset of symptoms. As can be readily seen in Fig. 1, two daily doses of 100 mg UA i.p. inhibited the onset of clinical signs of Borna disease and protected against a lethal outcome within this time frame. To determine whether UA treatment had any general effect on immunity, we assessed the levels of BDV-specific IgG Abs in the sera of BDV-infected rats treated with UA, allantoin, or saline. As shown in Fig. 2, all three groups of BDV-infected rats exhibited strong Ab responses to BDV. Nevertheless, UA-treated rats made somewhat less Ab ($p < 0.04$ by the paired t test) than either saline or allantoin controls (Fig. 2).

RNA expression in brain tissue from UA-treated and control BDV-infected rats

As UA treatment can prevent the development of CNS inflammation in a conventional mouse model of EAE (30), it seemed possible that a similar effect was operative in Borna disease. Nevertheless, it was also conceivable that the administration of UA had either a direct or indirect effect on the BDV infection, thereby leading to a reduced immune and inflammatory stimulus. To test these possibilities, we used real-time quantitative RT-PCR to assess the levels of mRNAs specific for CD4, CD8, and MHC class II in brains from UA- and saline vehicle-treated, BDV-infected rats (Fig. 3). In this experiment, UA treatment delayed the appearance and severity of clinical signs of BD, but when the tissues were taken (days 22–24 p.i.), the treated animals showed signs of disease (average 2 vs 3 in saline controls). Nevertheless, while the levels of BDV RNA were comparable in both groups of rats (data not shown), those of mRNA specific for CD4, CD8, and MHC class II were all significantly reduced by UA treatment ($p < 0.001$ by ANOVA) (Fig. 3). Similar results were obtained for IFN-γ and iNOS mRNA (data not shown). These results suggest that UA treatment has no effect on the replication of BDV, but inhibits the appearance in infected CNS tissue of CD4 and CD8 cells as well as activated cells of the monocyte lineage.

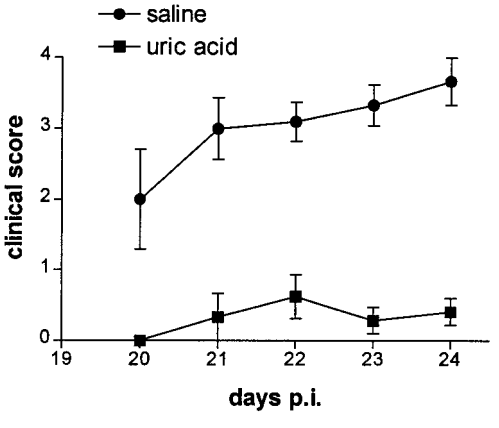

FIGURE 1. Effect of UA treatment on the development of clinical signs of Borna disease. Pooled results of three experiments are shown. In each, two groups of rats were infected with BDV intranasally and treated, beginning 14 days p.i., twice daily with either 100 mg UA in 1 ml saline i.p. or saline alone, as detailed in *Materials and Methods*. Clinical signs of Borna disease were recorded using a scale of 0–4, with 0 representing a healthy animal and 4 representing terminal Borna disease, as more fully described in *Materials and Methods*. Disease severity was significantly lower in the UA-treated animals ($p < 0.001$ by the t test).

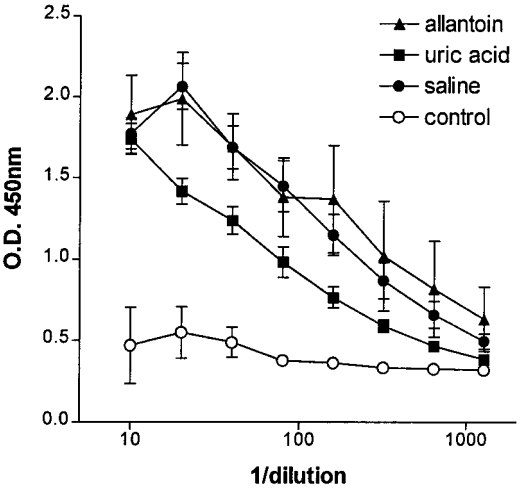

FIGURE 2. BDV-specific IgG Ab production in BDV-infected rats treated with UA, allantoin, and saline. Beginning 10 days p.i., groups of five to eight rats were treated twice daily i.p. with UA or allantoin (100 mg in 1 ml saline) or saline alone and bled 12–14 days afterward. BDV-specific IgG Ab levels were assessed in ELISA. Also shown is the reactivity of sera from four noninfected rats (control).

Immunohistochemical analysis of brains from control- and UA-treated, BDV-infected rats

To assess the effects of UA treatment on the distribution of BDV and immune/inflammatory cells in the infected brain, serial sections of brain tissue from control (saline)- and UA-treated rats infected with BDV 23 days previously were examined for BDV Ags as well as the inflammatory changes that normally accompany acute BDV disease. To assess whether UA treatment either prevented the spread of BDV through the CNS or promoted the clearance of the virus, sections from the brains of BDV-infected rats treated with either saline or UA were assessed for the presence of BDV Ag. High levels of BDV Ags were detected in brain tissue, regardless of whether UA had been administered, indicating that UA does not influence virus spread in the BDV-infected brain (Fig. 4, *B* and *C*). In the normal rat brain (Ctrl), very few inflammatory cells are seen by Giemsa (Fig. 4*D*) and ED1 immunostaining (Fig. 4*G*). Pronounced accumulations of inflammatory cells, stained with Giemsa (Fig. 4*E*) and ED1 (Fig. 4*H*), are found in the subarachnoid space, and in neocortical parenchyma of the BDV-infected brain (Fig. 4, *E* and *H*). UA treatment restricted Giemsa-stained inflammatory cells (Fig. 4*F*) and ED1-positive cells (Fig. 4*I*) to the subarachnoid space and blood vessels in the BDV-infected brain. In contrast, such cells are scarce in the brain parenchyma of UA-treated, BDV-infected rats. To assess the nature of the infiltrating cells in the BDV-infected rat brain as well as test for evidence of $ONOO^-$ formation, serial sections from the brains of control, BDV-infected, and UA-treated BDV-infected rats were stained with Abs specific for NT, iNOS, CD4, and CD8 (Fig. 5). Although there is little evidence of these markers in the uninfected control rat brain (Fig. 5, *A*, *D*, *G*, and *J*), they are extensively expressed throughout BDV-infected brain tissue (Fig. 5, *B*, *E*, *H*,

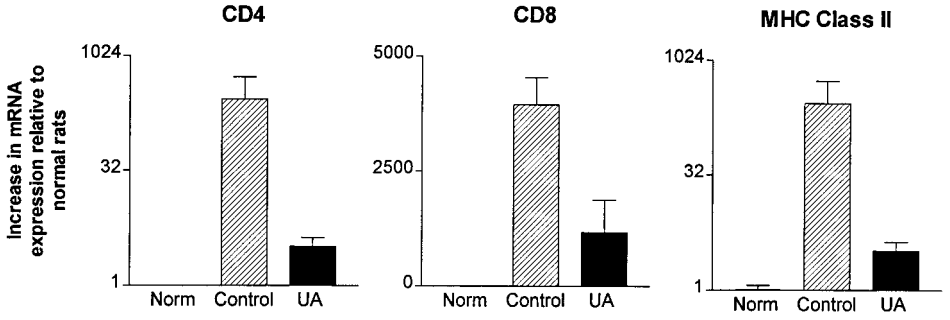

FIGURE 3. UA treatment inhibits the appearance of cells expressing mRNA specific for CD4, CD8, and MHC class II in the BDV-infected rat brain. Groups of four rats were infected with BDV and, beginning at day 10, treated twice daily i.p. with either 100 mg UA (UA) in 1 ml saline, or saline alone (Control). At 22–24 days p.i., treated animals and normal controls (Norm) were perfused, and RNA was isolated from the brains and subjected to quantitative PCR, as described in *Materials and Methods*. Results are expressed as the mean ± SEM fold increase in specific mRNA levels compared with those of normal uninfected rats, corrected for GAPDH mRNA levels.

and *K*). Cells staining for NT (Fig. 5*B*), iNOS (Fig. 5*E*), CD4 (Fig. 5*H*), and CD8 (Fig. 5*K*) are seen in the subarachnoid space as well as massive infiltration into the neocortical parenchyma. This pattern was considerably changed by UA treatment following BDV infection. In this case, moderate numbers of cells stained for NT (Fig. 5*C*), iNOS (Fig. 5*F*), CD4 (Fig. 5*I*), and CD8 (Fig. 5*L*) accumulate in the subarachnoid space and in blood vessels, but infiltration into the neocortical parenchyma is minimal. In general, CD4 and CD8 cells were restricted to regions containing NT and some evidence of iNOS expression (Fig. 5, *C*, *F*, *I*, and *L*).

As shown in Fig. 6, UA treatment also prevented the extensive tissue damage normally associated with acute Borna disease. In the BDV-infected rat, a marked degeneration and accumulation of inflammatory cells into the brain parenchyma are seen (Fig. 6*C*). The damage is most pronounced in the temporal lobe and the hippocampus, particularly in the pyramidal cell layer as well as in the dentate gyrus. The brain from a UA-treated, BDV-infected rat remained histologically normal with the exception of cells accumulating in the vasculature (Fig. 6*D*).

BBB permeability in Borna disease

The antiinflammatory effect of UA in Borna disease resembles its effect on cell infiltration in EAE (30). In the latter case, UA has been demonstrated to protect the blood-CNS barrier from permeability changes most likely induced by $ONOO^-$ (30). Since, like EAE, the CNS inflammatory response in acute Borna disease is associated with the production of $NO^{\cdot}$ (15), it seemed possible that BDV infection may also promote cell invasion through $ONOO^-$-mediated disruption of the BBB. We therefore determined whether the integrity of BBB becomes compromised in acute Borna disease. As shown in Fig. 7, the permeability of the BBB to Na-fluorescein was significantly enhanced in rats with clinical signs of Borna disease. UA treatment (100 mg twice daily), beginning 7–9 days before analysis, largely prevented the increased permeability of the BBB associated with Borna disease in the control animals.

Discussion

CNS inflammation can be inhibited by the administration of UA whether induced by infection of rats with BDV or an autoimmune response to myelin Ags in mice (see Figs. 4 and 5) (30). UA treatment of BDV-infected rats inhibits the accumulation of cells expressing iNOS, ED-1, CD4, and CD8 in infected brain tissue, as well as NT formation and CNS tissue damage. UA-mediated suppression of inflammatory cell invasion into the BDV-infected brain was associated with maintenance of normal BBB integrity and prevention of the onset of clinical Borna disease, which is most often lethal in our model. Together these findings suggest that the action of $ONOO^-$ on the BBB is an important stage in the invasion of inflammatory cells, including monocytes and T lymphocytes, into CNS tissues.

Although UA is an efficient inhibitor of certain chemical reactions associated with $ONOO^-$ in vitro (35), there is some speculation that its molecular targets in vivo may be radicals, such as $NO_2^{\cdot}$ and $CO_3^{\cdot -}$, formed by the rapid reaction of $ONOO^-$ with CO_2 (36). Whatever the case, UA interferes with reactivity associated with $ONOO^-$. Shortly after mice with preexisting clinical signs of EAE begin UA treatment, NT residues, generally accepted as evidence of $ONOO^-$ formation (e.g., 37–39), are reduced in areas of iNOS-positive inflammation in the spinal cord (30), indicating that UA inactivates $ONOO^-$, or its reactive intermediates, in vivo. This supports the hypothesis that the inhibitory effect of UA treatment on cell invasion into the CNS is also a consequence of the inactivation of $ONOO^-$, and that this molecule or its products are involved in providing inflammatory cells access to CNS tissues across the BBB. Because of the variety of chemical reactions that $ONOO^-$ can undergo, this could be through a number of different mechanisms, including up-regulation of adhesion molecules on the cerebral vasculature, physiological effects on endothelial cell tight junctions and other elements of the BBB, or reactive damage to the BBB. There is evidence that $ONOO^-$ may induce the expression of adhesion molecules on cultured endothelial cells (40), but we do not believe that this is the sole effect of $ONOO^-$ on the BBB because the permeability to molecules like UA and fluorescein is also increased (Fig. 7) (30). Rather, we expect that the enhanced permeability facilitates the invasion of cells both physically and by promoting contact with chemoattractive factors, such as chemokines, which are expressed in the BDV-infected rat brain in the absence of inflammation (41).

In both BDV-infected and UA-treated BDV-infected rats, the extent of CD4 and CD8 T cell invasion into brain tissue paralleled the extent of NT formation. Cells expressing iNOS were also found throughout BDV-infected, control-treated brain tissue. UA treatment resulted in substantial inhibition of the accumulation of cells positive for iNOS, CD4, and CD8 as well as NT staining following BDV infection. For the most part, staining for these markers was limited to the meningeal regions of the subarachnoid space with little penetration deeper into the tissues. However, iNOS-positive cells appeared to be disproportionately rare, by comparison with those positive for CD4 and CD8, in BDV-infected brain tissue from UA-treated rats. It is conceivable that NT formed under these conditions is the result of the activity of endothelial NOS, possibly stimulated by activated T cells, rather than

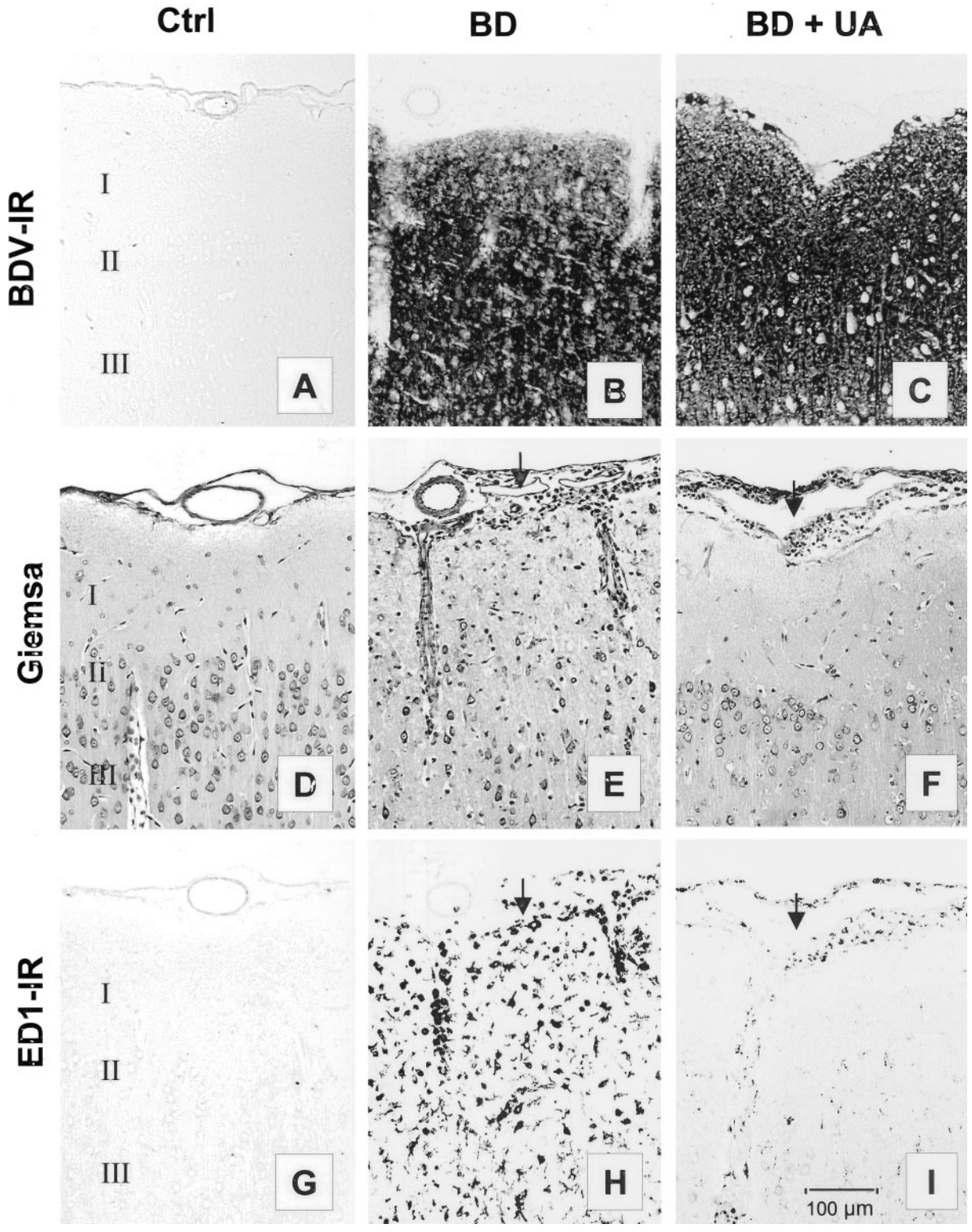

FIGURE 4. Effects of UA treatment on virus spread and inflammation in the parietal cortex of the BDV-infected rat brain. Adjacent brain sections from normal rats (Ctrl, *left column*, *A*, *D*, and *G*) and rats infected with BDV 23 days previously and treated from day 14 with saline (BD, *middle column*, *B*, *E*, and *H*) or UA (BD plus UA, *right column*, *C*, *F*, and *I*) were stained for BDV p38 Ag (BDV-IR) and activated cells of the monocyte lineage (ED1-IR). The general morphology and presence of inflammatory cells are shown by Giemsa staining. *I*, *II*, and *III* in the control panel (*A*, *D*, and *G*) denote the upper neocortical laminae (according to Ref. 34). Arrowheads, subarachnoid space.

iNOS. In this case, UA, which does not penetrate the intact BBB (30), may have limited access to the $ONOO^-$ produced by the endothelial cells, and some $ONOO^-$-dependent T cell invasion may still occur. The rats in this study received two daily doses of 100 mg UA, which caused serum UA levels to be elevated 4-fold, to an average of 2 mg/dl for ~4 h at a time. Although peritoneal macrophages may take up UA at the site of injection and carry it for several hours (42), the evidence of NT formation in brain tissue from UA-treated rats makes it seem unlikely that this treatment protocol provided sufficient UA to inactivate all of the $ONOO^-$

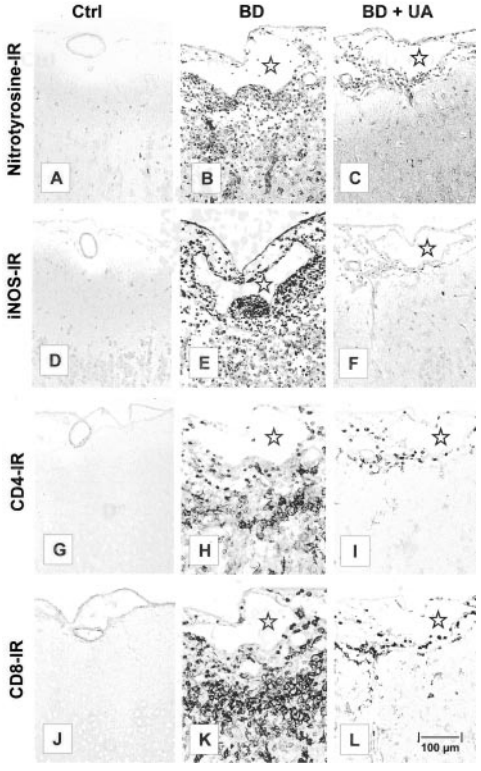

FIGURE 5. UA treatment reduces the appearance of iNOS-, CD4-, and CD8-positive cells and NT in the BDV-infected rat brain. Adjacent brain sections of parietal neocortex from a normal rat (Ctrl, *left column*, *A*, *D*, *G*, and *J*) and from rats infected with BDV 23 days previously and treated from day 14 with saline (BD, *middle column*, *B*, *E*, *H*, and *K*), or with UA (BD plus UA, *right column*, *C*, *F*, *I*, and *L*) were stained for NT (*A–C*), iNOS (*D–F*), and the T cell phenotype markers CD4 (*G–I*) and CD8 (*J–L*). The subarachnoid space is indicated by stars. Bar, 100 μm.

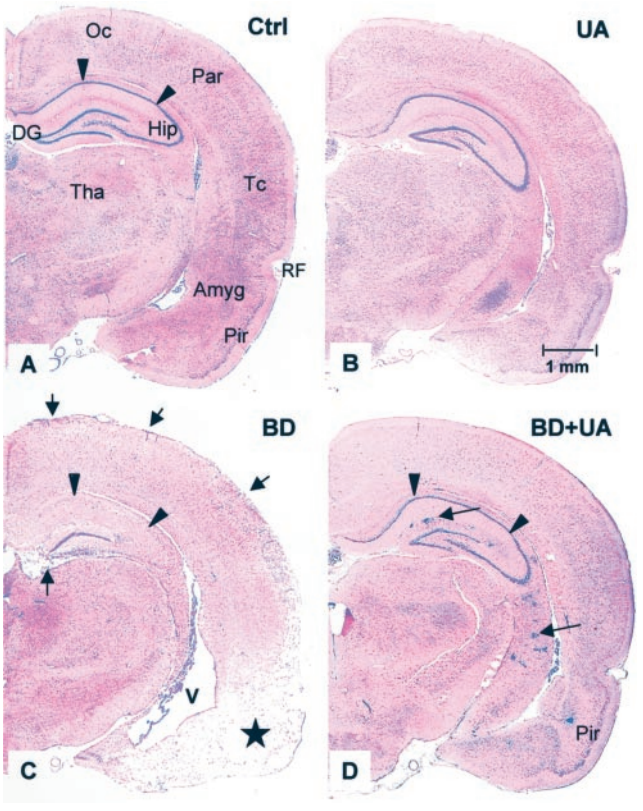

FIGURE 6. Effects of UA treatment on BDV-induced histopathological changes in rat brain. Giemsa-stained coronal brain sections from normal rats treated with saline (Ctrl, *A*) or UA (UA, *B*) and from BDV-infected rats treated from day 14 p.i. until day 21 p.i. with saline (BD, *C*) or UA (BD plus UA, *D*) were micrographed under low power. *C*, Accumulations of inflammatory cells in the brain parenchyma are indicated by arrows. Damage to the temporal lobe is denoted by a star. The hippocampus (Hip) is indicated by arrowheads. *D*, Inflammatory cells associated with blood vessels are indicated by arrows. DG, dentate gyrus; Oc, occipital cortex; Par, parietal cortex; Pir, piriform cortex; RF, rhinal fissure; Tc, temporal cortex; Tha, thalamus; V, ventricle; Amyg, Amygdala. Bar, 1 mm.

produced at the BBB. Regardless, the overlapping geographical distributions of CD4, CD8, and NT in the BDV-infected CNS, whether UA treated or not, suggest the possibility that $ONOO^-$ may make an important contribution to the invasion of T cells, as well as iNOS-positive cells, into CNS tissues.

Although our studies of $ONOO^-$ in EAE and Borna disease have largely focused on the contribution of this molecule to the pathogenic aspects of CNS inflammation, it is clear that, depending on the circumstances, $ONOO^-$-mediated processes may be protective. In situations in which a CNS inflammatory response contributes to recovery from a neurotropic infection, with BDV or other pathogens, $ONOO^-$ may participate in providing immune cells and Ab access to the focus of infection as well as in the destruction of the infecting agent and infected cells. In this regard, we speculate that the somewhat lowered Ab response to BDV seen in UA-treated rats (Fig. 2) may be due to reduced contact between the immune system and viral Ags as a consequence of maintained BBB integrity, lessened CNS inflammation, and diminished damage of infected neurons. It is conceivable that $ONOO^-$ makes contributions to protective immune responses against infected or transformed tissue in the periphery, similar to those seen in the CNS. Nevertheless, UA administration had no inhibitory effect on various parameters of Ag-specific immunity in mice immunized s.c. with myelin basic protein, which includes the appearance of iNOS-positive cells in the circulation (42, 43).

The demonstration that $ONOO^-$ participates in opening the BBB and providing inflammatory cells access to the CNS during a neurotropic infection in rodents has implications for immune responses to similar infections in humans. Most mammals, including mice and rats, possess urate oxidase, an enzyme that metabolizes UA, a product of purine metabolism, to allantoin, which does not inactivate $ONOO^-$ (35). Thus, serum levels of UA are low in most mammals, and can only be raised by repeated administration of

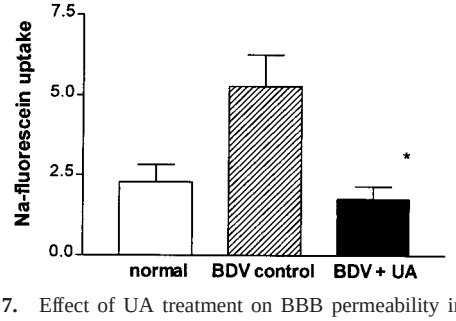

FIGURE 7. Effect of UA treatment on BBB permeability in BDV-infected rats. BDV-infected rats were treated with UA, allantoin, or saline, as described in *Materials and Methods*. Twenty-three days after infection, the rats and uninfected controls were given sodium-fluorescein i.v. and perfused, and brain tissue was assessed for the level of sodium-fluorescein. Levels of sodium-fluorescein in brain tissue from UA-treated BDV-infected rats were significantly lower than similarly infected rats that received allantoin and saline (BDV controls; $p < 0.001$ by the *t* test).

relatively large amounts of UA (26, 27). However, the urate oxidase gene has been inactivated in higher order primates, and their serum UA levels are substantially higher (44, 45). This has been the stimulus of considerable speculation as to the importance of UA to human evolution (e.g., 46–48). The fact that two daily doses of UA in rats are sufficient to protect CNS tissue from a robust immune response to BDV infection supports the notion that UA may play an important role in protecting the CNS, and possibly other tissues, from $ONOO^-$-dependent inflammatory cell invasion and damage. These data also imply that certain immune as well as pathogenic processes that involve $ONOO^-$ may be somewhat different in humans by comparison with lower animals. UA evidently does not directly interfere with the development of Ag-specific elements of the immune response to accessible Ags (42). Nevertheless, the relatively high levels of serum UA found in humans and higher order primates should limit $ONOO^-$-dependent BBB permeability changes as well as the invasion of immune and inflammatory cells into the CNS. Like BDV-infected rats treated with UA, high UA levels in humans may be expected to inhibit the invasion of monocytes and lymphocytes into the CNS in response to infection. Conceivably, UA may thereby contribute to the persistence of CNS infection with a virus of limited neuropathogenicity. An important example of this in humans may be HIV infection of the CNS. It is conceivable that serum UA levels may be relevant to the establishment of HIV infection in the brain and to the progression of the AIDS-dementia complex.

Acknowledgments

We thank C. Brimer, S. Fenn, E. Rosenberg-Frank, M. Zibuschka, P. Sack, and P. Lattermann for excellent technical assistance and H. Schneider for photography.

References

1. Rott, R., and H. Becht. 1995. Natural and experimental Borna disease in animals. *Curr. Top. Microbiol. Immunol. 190:17.*
2. Carbone, K. M., T. R. Moench, and W. I. Lipkin. 1991. Borna disease virus replicates in astrocytes, Schwann cells and ependymal cells in persistently infected rats: location of viral genomic and messenger RNAs by in situ hybridization. *J. Neuropathol. Exp. Neurol. 50:205.*
3. Sierra-Honigmann, A. M., S. A. Rubin, M. G. Estafanous, R. H. Yolken, and K. M. Carbone. 1993. Borna disease virus in peripheral blood mononuclear and bone marrow cells of neonatally and chronically infected rats. *J. Neuroimmunol. 45:31.*
4. Rubin, S. A., A. M. Sierra-Honigmann, H. M. Lederman, R. W. Waltrip II, J. J. Eiden, and K. M. Carbone. 1995. Hematologic consequences of Borna disease virus infection of rat bone marrow and thymus stromal cells. *Blood 85:2762.*
5. Stitz, L., B. Dietzschold, and K. M. Carbone. 1995. Immunopathogenesis of Borna disease. *Curr. Top. Microbiol. Immunol. 190:75.*
6. Herzog, S., K. Wonigeit, K. Frese, H. J. Hedrich, and R. Rott. 1985. Effect of Borna disease virus infection on athymic rats. *J. Gen. Virol. 66:503.*
7. Dittrich, W., L. Bode, H. Ludwig, M. Kao, and K. Schneider. 1989. Learning deficiencies in Borna disease virus-infected but clinically healthy rats. *Biol. Psychol. 26:818.*
8. Carbone, K. M., S. W. Park, S. A. Rubin, R. W. Waltrip, and G. B. Vogelsang. 1991. Borna disease: association with maturation defect in the cellular immune response. *J. Virol. 65:6154.*
9. Bautista, J. R., G. J. Schwartz, J. C. De La Torre, T. H. Moran, and K. M. Carbone. 1994. Early and persistent abnormalities in rats with neonatally-acquired Borna disease virus infection. *Brain Res. Bull. 34:31.*
10. Eisenman, L. M., R. Brothers, R. B. Kean, G. M. Dickson, B. Dietzschold, and D. C. Hooper. 1999. Neonatal Borna disease virus infection in the rat causes a loss of Purkinje cells in the cerebellum. *J. Neurovirol. 5:181.*
11. Richt, J. A., L. Stitz, H. Wekerle, and R. Rott. 1989. Borna disease, a progressive meningoencephalomyelitis as a model for $CD4^+$ T-cell-mediated immunopathology in the brain. *J. Exp. Med. 170:1045.*
12. Richt, J. A., L. Stitz, U. Deschl, K. Frese, and R. Rott. 1990. Borna disease virus-induced meningoencephalitis caused by a virus-specific $CD4^+$ T-cell mediated immune reaction. *J. Gen. Virol. 71:2565.*
13. Zheng, Y. M., M. K.-H. Schafer, E. Weihe, H. Sheng, S. Corisdeo, Z. F. Fu, H. Koprowski, and B. Dietzschold. 1993. Severity of neurological signs and degree of inflammatory lesions in the brain of rats with Borna disease correlate with the induction of nitric oxide synthase. *J. Virol. 67:5786.*
14. Planz, O., T. Bilzer, and L. Stitz. 1995. Immunopathogenic role of T-cell subsets in Borna disease virus-induced progressive encephalitis. *J. Virol. 69:896.*
15. Hooper, D. C., T. S. Ohnishi, R. Kean, Y. Numagami, B. Dietzschold, and H. Koprowski. 1995. Local nitric oxide production in viral and autoimmune diseases of the central nervous system. *Proc. Natl. Acad. Sci. USA 92:5312.*
16. Shankar, V., M. Kao, A. N. Hamir, H. Sheng, H. Koprowski, and B. Dietzschold. 1992. Kinetics of virus spread and changes in levels of several cytokine mRNAs in the brain after intranasal infection of rats with Borna disease virus. *J. Virol. 66:992.*
17. Dietzschold, B., W. Schwaeble, D. C. Hooper, M. K. Schafer, Y. M. Zheng, F. Petry, H. Sheng, T. Fink, M. Loos., H. Koprowski, and E. Weihe. 1995. The expression of C1q, a subcomponent of the rat complement system is dramatically enhanced in brains of rats with either Borna disease or experimental allergic encephalomyelitis. *J. Neurol. Sci. 130:11.*
18. Morimoto, K., D. C. Hooper, A. Bornhorst, S. Corrisdeo, M. Bette, Z. F. Fu, M. K. Schafer, E. Weihe, and B. Dietzschold. 1996. Intrinsic responses to Borna disease virus infection of the central nervous system. *Proc. Natl. Acad. Sci. USA 93:13345.*
19. Röhrenbeck, A. M., M. Bette, D. C. Hooper, F. Nyberg, L. E. Eiden, B. Dietzschold, and E. Weihe. 1999. Up-regulation of COX-2 and CGRP expression in resident cells of the Borna disease virus-infected brain is dependent upon inflammation. *Neurobiol. Dis. 6:15.*
20. Rall, G. F. 1998. CNS neurons: the basis and benefits of low class I major histocompatibility complex expression. *Curr. Top. Microbiol. Immunol. 232:115.*
21. Beckman, J. S. 1991. The double-edged role of nitric oxide in brain function and superoxide-mediated injury. *J. Dev. Physiol. 15:53.*
22. Lin, R. F., T.-S. Lin, R. G. Tilton, and A. H. Cross. 1993. Nitric oxide localized to the spinal cords of mice with experimental allergic encephalomyelitis: an electron paramagnetic resonance study. *J. Exp. Med. 178:643.*
23. Koprowski, H., Y. M. Zhang, E. Heber-Katz, N. Fraser, L. Rorke, Z. F. Fu, C. Hanlon, and B. Dietzschold. 1993. In vivo expression of inducible nitric oxide synthase in experimentally induced neurologic diseases. *Proc. Natl. Acad. Sci. USA 90:3024.*
24. Beckman, J. 1994. Peroxynitrite versus hydroxyl radical: the role of nitric oxide in superoxide-dependent cerebral injury. *Ann. NY Acad. Sci. 738:69.*
25. Squadrito, G. L., and W. A. Pryor. 1995. The formation of $ONOO^-$ in vivo from nitric oxide and superoxide. *Chem. Biol. Interact. 96:203.*
26. Hooper, D. C., O. Bagasra, J. C. Marini, A. Zborek, S. T. Ohnishi, R. Kean, J. M. Champion, A. B. Sarker, L. Bobroski, J. L. Farber, et al. 1997. Prevention of experimental allergic encephalomyelitis by targeting nitric oxide and $ONOO^-$: implications for the treatment of multiple sclerosis. *Proc. Natl. Acad. Sci. USA 94:2528.*
27. Hooper, D. C., S. V. Spitsin, R. B. Kean, J. M. Champion, G. M. Dickson, I. Chaundry, and H. Koprowski. 1998. Uric acid, a natural scavenger of $ONOO^-$, in experimental allergic encephalomyelitis and multiple sclerosis. *Proc. Natl. Acad. Sci. USA 95:675.*
28. Denicola, A., B. A. Freeman, M. Trujillo, and R. Radi. 1996. $ONOO^-$ reaction with carbon dioxide/bicarbonate: kinetics and influence on $ONOO^-$-mediated oxidations. *Arch. Biochem. Biophys. 333:49.*
29. Uppu, R. M., G. L. Squadrito, and W. A. Pryor. 1996. Acceleration of $ONOO^-$ oxidations by carbon dioxide. *Arch. Biochem. Biophys. 327:335.*
30. Hooper, D. C., G. S. Scott, A. Zborek, T. Mikheeva, R. B. Kean, H. Koprowski, and S. V. Spitsin. 2000. Uric acid, a peroxynitrite scavenger, inhibits CNS inflammation, blood-CNS barrier permeability changes, and tissue damage in a mouse model of multiple sclerosis. *FASEB J. 14:691.*
31. Uttenthal, L. O., D. Alonso, A. P. Fernandez, R. O. Campbell, M. A. Moro, J. C. Leza, I. Lizasoain, F. J. Esteban, J. B. Barroso, R. Valderrama, et al. 1998. Neuronal and inducible nitric oxide synthase and nitrotyrosine immunoreactivities in the cerebral cortex of the aging rat. *Microsc. Res. Tech. 43:75.*
32. Müller, S., and E. Weihe. 1991. Interrelation of peptidergic innervation with mast cells and ED1-positive cells in rat thymus. *Brain Behav. Immun. 5:55.*
33. Zentel, H. J., and E. Weihe. 1991. The neuro-B cell link of peptidergic innervation in the bursa fabricii. *Brain Behav. Immun. 5:132.*
34. Zilles, K., and A. Wree. 1985. Cortex: areal and laminar structure. In *The Rat Nervous System: Forebrain and Midbrain*, Vol. 1. G. Paxinos, ed. Academic, pp. 375–415.
35. Whiteman, M., and B. Halliwell. 1996. Protection against $ONOO^-$-dependent tyrosine nitration and α_1-antiproteinase inactivation by ascorbic acid: a comparison with other biological antioxidants. *Free Radical Res. 25:275.*
36. Squadrito, G. L., R. Cueto, A. E. Splesner, A. Valavanidis, H. Zhang, R. M. Uppu, and W. A. Pryor. 2000. Reaction of uric acid with peroxynitrite and implications for the mechanism of neuroprotection. *Arch. Biochem. Biophys. 376: 333.*
37. Ischiropoulos, H., L. Zhu, and J. S. Beckman. 1992. Peroxynitrite formation from macrophage-derived nitric oxide. *Arch. Biochem. Biophys. 298:446.*
38. Van der Veen, R. C., D. R. Hinton, F. Incardonna, and F. M. Hofman. 1997. Extensive $ONOO^-$ activity during progressive stages of central nervous system inflammation. *J. Neuroimmunol. 77:1.*
39. Cross, A. H., P. T. Manning, M. K. Stern, and T. P. Misko. 1997. Evidence for the production of $ONOO^-$ in inflammatory CNS demyelination. *J. Neuroimmunol. 80:121.*

40. Zingarelli, B., A. L. Salzman, and C. Szabó. 1998. Genetic disruption of poly(ADP-ribose) synthetase inhibits the expression of P-selection and intercellular adhesion molecule-1 in myocardial ischemia/reperfusion injury. *Circ. Res. 83:83.*
41. Sauder, C., W. Hallensleben, A. Pagenstecher, S. Schneckenburger, L. Biro, D. Pertlik, J. Hausmann, M. Suter, and P. Staeheli. 2000. Chemokine gene expression in astrocytes of Borna disease virus-infected rats and mice in the absence of inflammation. *J. Virol. 74:9267.*
42. Kean, R. B., S. V. Spitsin, T. Mikheeva, G. S. Scott, and D. C. Hooper. 2000. 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. *J. Immunol. 165:6511.*
43. Spitsin, S. V., G. S. Scott, R. B. Kean, T. Mikheeva, and D. C. Hooper. 2000. Protection of myelin basic protein immunized mice from free-radical mediated inflammatory cell invasion of the central nervous system by the natural peroxynitrite scavenger uric acid. *Neurosci. Lett. 292:137.*
44. Yelandi, A. V., V. Yelandi, S. Kumar, C. V. Murthy, X. D. Wang, K. Alvares, M. S. Roa, and J. K. Reddy. 1991. Molecular evolution of the urate oxidase-encoding gene in hominid primates: nonsense mutations. *Gene 109:281.*
45. Wu, X. W., D. M. Muzny, C. C. Lee, and C. T. Catskey. 1992. Two independent mutational events in the loss of urate oxidase during hominid evolution. *J. Mol. Evol. 34:78.*
46. Proctor, P. 1970. Similar functions of uric acid and ascorbate in man? *Nature 228:868.*
47. Ames, B. N., R. Cathcart, E. Schwiers, and P. Hochstein. 1981. Uric acid provides an antioxidant defense in humans against oxidant- and radical-caused aging and cancer: a hypothesis. *Proc. Natl. Acad. Sci. USA 78:6858.*
48. Scott, G. S., and D. C. Hooper. 2001. The role of uric acid in protecting the central nervous system from peroxynitrite-mediated pathology. *Med. Hypotheses 56:95.*