Nitric Oxide Contributes to Resistance of the Brown Norway Rat to Experimental Autoimmune Encephalomyelitis

Maria A. Staykova,* Judith T. Paridaen,[†]
William B. Cowden,[‡] and David O. Willenborg*[§]

From the Neurosciences Research Unit,* The Canberra Hospital, Canberra, Australia; the Faculty of Medicine, University of Utrecht,[†] Utrecht, The Netherlands; The John Curtin School of Medical Research,[‡] Australian National University, Canberra; and The Australian National University School of Medicine,[§] Canberra, Australia

The Brown Norway (BN) rat is reported to be resistant to the induction of experimental autoimmune encephalomyelitis (EAE) and a number of mechanisms have been suggested to explain this resistance. In work reported here we provide evidence that such resistance in the BN rat can be accounted for, at least in part, by their ability to produce higher levels of nitric oxide (NO) than susceptible strains of rats. Spleen cells from the BN rat make significantly more NO following *in vitro* stimulation than do cells from the Lewis or PVG rat and following *in vivo* immunization using complete Freund's adjuvant (CFA) the BN rat makes substantially more NO than either susceptible strain. If carbonyl iron is used as adjuvant *in vivo* there is no increase in NO levels in the BN rat and they are rendered highly susceptible to EAE. Immunizing with CFA simultaneously with neuroantigen and carbonyl iron drives up NO levels and the resistance is restored. EAE produced using carbonyl iron is characterized by extensive macrophage/microglia presence in the central nervous system lesions of the BN rat yet the cytokine profile in the lymph nodes does not differ from that in the EAE Lewis rats. (*Am J Pathol* 2005, 166:147–157)

Nitric oxide (NO) is a free radical produced from the oxidation of the terminal guanidino nitrogen of arginine; the reaction is catalyzed by an NADPH-dependent enzyme, nitric oxide synthase (NOS) of which three isoforms have been described.^{1–3} The inducible form of the enzyme (iNOS) is up-regulated during inflammation and

infection and up-regulation can be sustained over a prolonged period culminating in the production of large quantities of NO.² High levels of NO have been implicated in the pathogenesis and tissue damage found in a number of disease states,⁴ including inflammatory autoimmune diseases. There have been, for example, numerous studies in the last few years that have focused on the role NO plays in the central nervous system (CNS) inflammatory disease autoimmune experimental encephalomyelitis (EAE) (for review, see Willenborg et al⁵).

Results from much of the early work on NO and EAE,^{6–9} including our own,¹⁰ suggested a deleterious effect of NO in this CNS inflammation. Later studies, however, began to shift the focus from the idea of NO as a “bad” molecule to one in which it plays an important role in down-regulating the disease process.⁵ We¹¹ and others^{12,13} showed that inhibition of NO production by treatment of Lewis rats with nitric oxide synthase (NOS) inhibitors enhances actively induced EAE. We further demonstrated that the EAE-resistant PVG rat produces up to four times higher serum reactive nitrogen intermediates (RNI) levels than Lewis rats following immunization with myelin basic protein with complete Freund's adjuvant (MBP-CFA) and that treatment of immunized PVG rats with the potent NOS inhibitor, *N*-methyl-L-arginine (L-NMA), rendered them highly susceptible to disease induction.¹¹ We also showed that the major source of NO in the PVG rat was most likely the spleen in that splenectomy completely abrogated the increased serum RNI levels following immunization of the PVG rat, and again rendered them highly susceptible to EAE.¹⁴

Recently, other studies have demonstrated that the acute monophasic nature of EAE in the Lewis rat as well as the Lewis rat's resistance to re-induction of disease

Supported by grants from Multiple Sclerosis Australia and the National Health and Medical Research Council (NH&MRC) of Australia as well as the Private Practice Fund of The Canberra Hospital. D.O.W. is a Fellow of the NH&MRC.

Accepted for publication August 9, 2004.

Address reprint requests to Maria A. Staykova, Neurosciences Research Unit, The Canberra Hospital, Canberra, Australia. E-mail: maria.staykova@anu.edu.au.

following recovery can also be accounted for, at least in part, by the increased production of NO.^{15,16} Rats recovering from MBP-CFA-induced EAE have significantly increased levels of serum RNI, indicative of increased NO production. These levels remain elevated after the recovery period and increase even further early after a re-challenge with MBP-CFA. These animals are totally refractory to a second episode of disease.¹⁷ Oral treatment of rats with L-NMA beginning at peak disease prolongs the disease episode until the drug is stopped. Treatment of fully recovered rats with L-NMA beginning 24 hours before a re-challenge with MBP-CFA leads to decreased serum RNI levels and results in a second episode of EAE in 100% of animals. In point of fact, treatment of recovered Lewis rats with L-NMA even in the absence of re-challenge with antigen results in a secondary episode of disease.¹⁶ These data point to NO as a potent regulator of this autoimmune response.

The Brown Norway (BN) rat has long been regarded as "resistant" to EAE^{18–20} and this resistance has been mapped to both MHC and non-MHC regions of the genome.^{18,21,22} Recently, studies on the BN rat have described the BN as mounting a Th2 type of immune response and a number of non-MHC regulated mechanisms have been suggested, such as endogenous corticosteroid levels,²³ enhanced TGF β production,^{24,25} and CD8 bias.²⁶ Here we re-examine the resistance of the BN rat to EAE in light of our current knowledge of the role NO can play in regulation of this disease. We report experiments in which we demonstrate both *in vitro* and *in vivo* that the BN rat produces significantly more NO (as reactive nitrogen intermediates, RNI) following stimulation or immunization with neuroantigen and complete Freund's adjuvant (CFA) than either the PVG or Lewis rat. Using the unique adjuvant carbonyl iron (CI), as originally described by Levine and Sowinski,²⁰ we are able to induce EAE in BN rats and we further demonstrate that with this adjuvant these rats do not increase their NO production following immunization. If complete Freund's adjuvant is administered simultaneously with carbonyl iron and neuroantigen, the BN rats produce high levels of NO and fail to develop EAE. The pathology in the BN rat immunized using CI is unique in that the lesions show an extensive infiltration of macrophages/activated microglia. The cytokine profiles of both inflamed spinal cord and draining lymph nodes do not differ between Lewis rats immunized with neuroantigen in CFA and BN rats immunized with neuroantigen plus CI, suggesting both responses are Th1 responses. These results once more point to the important role of the innate immune system, and in particular NO, in immune regulation.

Materials and Methods

Animals

Lewis and PVG rats were bred by the Animal Breeding Establishment of the John Curtin School of Medical Research, Australian National University. Breeding pairs of BN rats were obtained from the Animal Resource Centre,

Canning Vale, Western Australia, and bred on the premises at the Canberra Hospital. The use of animals and all procedures performed were approved by the Animal Experimentation Ethics Committee of the Australian National University following the guidelines of the National Health and Medical Research Council of Australia.

Immunizations

MBP was purified from frozen guinea pig spinal cord according to the method of Eylar et al.²⁷ MBP in saline was emulsified in an equal volume of incomplete Freund's adjuvant with added *Mycobacterium butyricum* (Bacto Laboratories, Liverpool, Australia) (4 mg/ml). Rats received 100- μ l emulsion in each hind footpad. When using rat spinal cord homogenates (SCH) the original procedure of Levine and Sowinski²⁰ was followed. Frozen spinal cords were passed through a mesh and 40% homogenates in saline were left in a 60°C water bath for 1 hour. SCHs were either emulsified with equal volume of CFA (containing 4 mg/ml *Mycobacterium tuberculosis*) and injected at a total of 100 μ l per rat (50 μ l in each hind footpad) or mixed with CI (Sigma, 100 mg CI:1 ml SCH) and 50 μ l injected into one hind footpad.

Evaluation of EAE Clinical Signs

Rats were examined on a daily basis and clinical scores recorded from day 5 to termination of the experiment. Clinical disease severity was assessed and scored as previously described¹⁷ using a scale from 1 to 5 as follows: 0, asymptomatic; 1, flaccid distal half of tail; 2, entire tail flaccid; 3, ataxia, difficulty in righting; 4, hind limb weakness; 5, hind limb paralysis. Body weight was also recorded in some experiments.

Measurement of Nitric Oxide Production

The levels of nitrate and nitrite in serum, urine, and cell culture samples were determined as an indirect measurement of NO production (described in detail by Cowden et al).¹¹ Briefly, 30- μ l aliquots were added in duplicate to a V-bottomed plates (Nunc). Standard curves were generated using culture media or dialysed rat serum to which sodium nitrite or sodium nitrate had been added at concentrations ranging from 1 mmol/L to 1 μ mol/L. To measure nitrate, the addition of nitrate reductase and NADPH (Boehringer Mannheim, Mannheim, Germany) for 30 minutes is required for conversion to nitrite. Nitrite was measured by addition of Griess reagent. Trichloroacetic acid was added to precipitate protein, the plates were centrifuged and the optical density of each supernatant was read at 540 nm with a reference wavelength of 650 nm using a Multiscan Platereader (Thermo Labsystems). Nitrate and nitrite levels were quantified by reading against the appropriate standard curves. The results were expressed as micromolar (μ M) concentrations of reactive nitrogen intermediates (RNI) ie, the sum of nitrate and nitrite concentrations.

In Vitro Cytokine Stimulation of Spleen Cells

Spleen cell suspensions were cultured at a concentration of 2×10^5 per well in 96-well plates in DMEM medium with 10% fetal calf serum (FCS), 2ME, and L-glutamine. Cultures were set up in the absence or presence of 10 U/ml rat recombinant IFN γ (GIBCO BRL) for 48 hours.

Immunopathology and Immunohistochemistry

Two centimeters of spinal cord encompassing the lumbar enlargement were taken from each of three animals and sectioned longitudinally at three different levels through the cord. Sets of six serial sections were cut and three sections (every other one) were mounted on each of two slides. This was repeated three times leaving six sections between sets. This was repeated at three levels through the cord. Half the slides were stained with H&E and the other used for immunohistochemistry (see below). A total of 27 sections were examined. Lesions were assessed semi-quantitatively and were scored 0 to +++, with 0, no lesions; +, one lesion in an occasional low-power field; ++, one lesion in almost every field; and +++, several lesions in many fields.

Serial paraffin sections (7 μ m) of lumbar spinal cord (as above), lymph nodes, and spleens were subjected to a 20-minute antigen retrieval in citrate buffer (pH 6.0) at 95°C. Peritoneal exudate cells were harvested 3 days after an i.p. injection of 100 μ l SCH-CFA or 50 μ l SCH-CI. Slides of peritoneal exudate cells were prepared by cytospin and fixed with acetone. The endogenous peroxidase activity and the non-specific antibody binding were blocked by incubation with peroxide block and power block (InnoGenex Immunohistochemistry Kit, San Remon, CA). Biotinylated mouse anti-rat ED1 monoclonal antibody (Serotec, Oxford, England) and rabbit anti-rat iNOS polyclonal antiserum (Sapphire Bioscience, Australia) were used as primary antibodies. The secondary antibodies and the developing were according to the manufacturer's instructions (InnoGenex). The sections were counterstained with Mayer's hematoxylin and mounted in SuperMount (Sapphire Bioscience).

PCR Analysis of Cytokines

On different days after immunization with SCH-CFA or SCH-CI Lewis and BN rats were anesthetized, perfused with cold phosphate-buffered saline (PBS), and the popliteal lymph nodes and lumbar spinal cords were stored in RNazol at -70°C. mRNA was extracted and transcribed into cDNA using reverse transcriptase (Qiagen, Victoria, Australia). Specific cytokine cDNA fragments were amplified with *Taq* polymerase (Epicenter, Madison, WI) in FailSafe buffer E (for IL2 and IFN γ), and buffer D (for TNF α , IL4, and IL10). Amplified PCR products were run in 2.3% agarose gel. The sequences of primer pairs (synthesized by GeneWorks, Australia) were as follows:

β -actin: sense 5'-CTATCGGCAATGAGCGGTTC-3' and antisense 5'-CTTAGGAGTTGGGGTGGCT-3';

Table 1. RNI Levels in Culture Supernatants of IFN γ -Stimulated Spleen Cells from Three Rat Strains

Rat strain	RNI (μ M) Unstimulated cells	RNI (μ M) IFN γ -stimulated cells
Lewis	11.4 $\pm$ 2	36.5 $\pm$ 4.3
PVG	5.6 $\pm$ 2.1	100.2 $\pm$ 4.6*
BN	3.7 $\pm$ 1.2	200.8 $\pm$ 10.3*†

Five animals per group. Mean $\pm$ standard deviation, $P < 0.001$ in comparison with the Lewis (*) and PVG (†).

IL-2: sense 5'-CCACTTCAAGCCCTGCAAAGGA-3' and antisense 5'-GACAGATGGCTATCCATCTCCTCAG-3'; IFN γ : sense 5'-TGAACGCTACACACTGCATCTTGG-3' and antisense 5'-CGACTCCTTTTCCGCTTCCTGAG-3'; TNF α : sense 5'-TCGAGTGACAAGCCCGTAG-3' and antisense 5'-GGCAGAGAGGAGGCTGACTT-3'; IL4: sense 5'-TCCATGCACCGAGATGTTGTACC-3' and antisense 5'-CTTTCAGTGTTGTGAGCGTGGACT-3'; IL10: sense 5'-TAAGGTTACCTTGGGTTGCCAAGCC-3' and antisense 5'-AGGGGAGAAATCGATGACAGCGCC-3'.

The intensities of the bands of the amplified PCR products were determined by densitometry and values have been normalized to that of β -actin and expressed as percentage.

Statistics

The analysis was done with Student's *t*-test or analysis of variance with Dunnett's Post-Hoc Test.

Results

In Vitro RNI Production by Lewis, PVG, and BN Rat Splenocytes

Spleen cells from groups of non-immunized age-matched female Lewis, PVG, and BN rats were cultured *in vitro* with or without IFN γ . Supernatants were assayed for RNI 48 hours later. The results (Table 1) demonstrate that PVG splenocytes produce approximately 2.5 times the amount of RNI as the Lewis splenocytes and the BN splenocytes twice that of the PVG and five times that of the Lewis cells. Thus, with respect to the *in vitro* RNI production by splenocytes: BN>PVG>Lewis.

In Vivo Production of RNI and Their Excretion in the Urine by the Three Strains of Rats following MBP-CFA Immunization

The *in vivo* levels of serum RNI seen in the three rat strains following immunization with MBP-CFA do not in fact parallel the *in vitro* levels of RNI produced by spleen cells

Figure 1. RNI levels in the serum (A) of PVG (hashed bars) and BN (black bars) rats following immunization with MBP-CFA are significantly increased when compared to the levels in Lewis rats (white bars) (significance of 0.006, analysis of variance Post-Hoc Test) but the BN levels do not differ from the PVG (except at day 1 where they are in fact lower). RNI levels in the urine (B) of the BN are significantly greater than either the Lewis or PVG (significance of 0.006, analysis of variance Post-Hoc Test) suggesting that the BN rat does make higher levels than the PVG but the RNI are excreted (5 animals/group/time period).

following IFN γ stimulation. The PVG rat is relatively resistant to EAE induction compared to the Lewis rat and following immunization produces up to three times higher serum RNI levels than the Lewis rat; this being similar to the *in vitro* situation. The BN rat, however, is strongly resistant to MBP-CFA-induced EAE, their spleen cells produce twice the amount of RNI as PVG cells, yet *in vivo*, following immunization, the BN serum RNI levels are only equal to or even slightly lower than the PVG (Figure 1A). Furthermore, oral treatment of immunized PVG rats with the NOS inhibitor *N*-methyl-L-arginine (L-NMA) has been shown to reduce the RNI levels and render the rats highly susceptible to EAE induction,¹¹ but interestingly, we have been unable to lower the serum RNI levels of the immunized BN rat with non-toxic doses of NOS inhibitors or to convert the BN to EAE susceptibility with such NOS inhibitors (data not shown). The puzzle of the BN splenocytes making significantly more RNI in culture than the PVG splenocytes, yet this difference not being reflected in the serum RNI levels following *in vivo* immunization, and our inability to lower serum levels with drugs, suggested

to us the possibility that the BN rat may in fact be making more RNI *in vivo* than the PVG rat but excreting them in the urine at a faster rate. As seen in Figure 1B, this would appear to be the case as the BN rats excreted up to three times more RNI in the urine than the PVG on a background of equal serum levels.

EAE Induction and RNI Levels in Lewis and BN Rats Immunized with Spinal Cord Homogenate Plus Carbonyl Iron as Adjuvant: Comparison with CFA

The increased production and maintenance of high levels of RNI in the BN rat prevented our assessing the role of NO in the resistance of the BN to EAE by the use of NOS inhibitors. We therefore revisited the original experiments of Levine and Sowinski,²⁰ who showed that the BN rat could develop severe EAE if carbonyl iron was used as adjuvant. In doing so we asked if one possible mechanism for the efficacy of carbonyl iron was that it did not induce NO production in the BN rat following immunization. Soluble MBP could not be used with carbonyl iron as the two dissociate too rapidly and therefore we used spinal cord homogenate (SCH) as did Levine and Sowinski.²⁰ The EAE susceptibility profile of the two rat strains is the same with SCH-CFA as with MBP-CFA, ie, Lewis>BN (Table 2). Carbonyl iron as adjuvant in the Lewis rat had the effect of increasing the severity and duration of disease when compared with CFA as adjuvant, though the increase in duration was not significant. Carbonyl iron usage in the BN rat significantly increased the incidence of disease and the disease produced was quite severe. Interestingly, the duration of disease in the BN immunized with CI was significantly longer than the CI-induced disease in the Lewis rat (Table 2). In another experiment (Figure 2A), we demonstrate that there is no disease and no weight loss in BN rats immunized with SCH-CFA and this on a background of elevated serum RNI levels. Rats immunized with SCH-carbonyl iron (Figure 2B) show no increase of serum RNI levels and develop severe disease with significant weight loss.

Table 2. Clinical EAE in Lewis and Brown Norway Rats Immunized with Spinal Cord Homogenate Using Either CFA or Carbonyl Iron as Adjuvant

Rat strain	Immunization	Clinical EAE			
		Incidence (%)	Max clinical score (individual)	Day of onset	Duration (days)
Lewis	SCH-CFA	5/6 (83%)	0 2, 2, 3, 3, 4	14 $\pm$ 0.2	4.3 $\pm$ 0.5
	SCH-carbonyl iron	8/9 (89%)	0, 2, 4, 4, 4, 4, 4, 4, 5	13.9 $\pm$ 3.6	6.3 $\pm$ 2.9
BN	SCH-CFA	1/8 (13%)	0, 0, 0, 0, 0, 0, 0 1	10	4
	SCH-carbonyl iron	9/13 (69%)	0, 0, 0, 0, 1, 3, 4, 4, 4, 4, 4, 5, 5	14.7 $\pm$ 2.3	12.1 $\pm$ 3.4*

Mean $\pm$ SD, *, $P < 0.01$ in comparison with the Lewis groups.

Figure 2. No clinical disease (black circle) and no change in body weight (gray circle) is seen in the BN rats immunized with SCH-CFA (A) but there is an increase in the serum RNI levels (histogram) (7 rats/group). Following immunization with SCH-carbonyl iron, however, (B) there is no increase in the serum RNI levels and the animals develop severe EAE accompanied by significant weight loss (11 rats/group).

Comparison of iNOS Expression in Draining Lymph Nodes, Spleen, and Peritoneal Exudate Cells from SCH-CFA- and SCH-CI-Immunized BN Rats

We also examined the difference in NO production by various BN rat tissues following immunization with either CFA or with carbonyl iron to determine whether increased tissue iNOS might reflect serum RNI levels following CFA. To do this we performed immunohistochemistry for iNOS expression in draining lymph nodes and spleen taken 7 days after immunization in the foot pads with SCH and either adjuvant. As shown in Figure 3, A and B, CFA immunization led to intense expression of iNOS in draining lymph nodes and spleen whereas immunization with carbonyl iron (Figure 3, D and E) showed much less staining for iNOS in these tissues. We also injected SCH-CFA or SCH-CI directly into the peritoneal cavity and exudate was harvested 3 days later. Cells from animals stimulated with CFA (Figure 3C) exhibited much greater iNOS staining than cells from animals stimulated with CI (Figure 3F). Carbonyl iron particles can be seen in the latter photo (arrows). Thus, CFA drives the production of iNOS whereas carbonyl iron does not.

Co-Immunization with CFA Along with SCH-Carbonyl Iron Increases Serum RNI Levels and Abrogates the Ability of Carbonyl Iron to Induce Disease

To further demonstrate the role for NO in resistance of the BN rat we increased NO production by immunizing with CFA at the same time as SCH-CI. BN rats were immunized with SCH-CI plus CFA in one hind footpad. Positive-control rats received only SCH-CI in one footpad and negative-control rats received only SCH-CFA in one footpad. The animals were observed for clinical disease and serum RNI levels were measured at a single time point (day 14 post-immunization). As shown in Table 3, SCH-CFA-immunized rats did not develop clinical EAE whereas the SCH-CI animals did. As before, serum RNI levels were two to three times higher (32 to 46 $\mu\text{mol/L}$) in CFA-immunized rats than in carbonyl iron-immunized rats (11 to 14 $\mu\text{mol/L}$). Rats given both CFA and carbonyl iron had serum RNI levels similar to those given CFA alone, failed to develop clinical EAE, and showed only marginal weight loss. Similar results were obtained when the CFA was given in one hind footpad and SCH-carbonyl iron in the other (data not shown).

Histopathology and Immunohistochemistry of EAE in the Lewis and BN Rat Immunized with SCH-Carbonyl Iron

When immunized with SCH-CFA, only the Lewis rats developed clinical EAE and Figure 4A shows a typical spinal cord meningeal lesion from a rat with clinical score of 3. Figure 4E shows a similar area of spinal cord meninges of a BN rat immunized with SCH-CFA for comparison and demonstrates that there are no lesions in these clinically disease-free animals. SCH-CI induced EAE in both Lewis and BN rats as demonstrated above. At one and the same clinical score the extent and the intensity of the inflammatory lesions in the lumbar spinal cord were essentially the same in the Lewis rats immunized with SCH-CFA and SCH-CI and in the BN rats immunized with SCH-CI. The lesion load at three levels of the lower lumbar spinal cord in three animals of each of the above three groups was: ++ in the meninges; +++ in the white matter; + to ++ in the gray matter. There were no differences between the groups. Typical pictures after H&E staining are shown in Figure 4, B to D-Lewis and 4, F to H-BN.

Table 3. Co-Immunization with CFA and SCH-Carbonyl Iron Increases RNI Production and Abrogates the Ability of Carbonyl Iron to Induce Disease

Immunization	BN rats (number)	Weight loss (%)	Maximal clinical score	Serum RNI (μM)
SCH-CFA	2	0, 0	0, 0	46.7, 32.1
SCH-carbonyl iron	2	10, 11	4, 4	11.2, 13.9
SCH-carbonyl iron + CFA	4	0, 0, 1, 2	0, 0, 0, 0	48.3, 32.6, 39.5, 25.1

Figure 3. Immunohistochemistry demonstrating iNOS expression in draining lymph nodes (**A** and **D**), spleen (**B** and **E**), and peritoneal exudate cells (**C** and **F**) of BN rats immunized with either SCH-CFA (**top row**) or SCH-CI (**bottom row**). The iNOS positivity in the tissues is brown-orange while in the cytoplasm of peritoneal cells it is brown-pink. **Arrows** in **F** point to carbonyl iron particles. Magnification, $\times 25$.

Figure 4. Histopathology (H&E counterstain) of lumbar spinal cord from Lewis (**A-D**) and BN (**E-H**) rats immunized with either SCH-CFA (**A** and **E**) or SCH-CI (**B** to **D** and **F** to **H**). Lewis rats killed at day 12 after immunization with SCH-CFA have severe lesions in many parts of the spinal cord. Only the meninges are shown here (**A**). For comparison, BN rats immunized also with SCH-CFA have no disease and show no lesions in the meninges (**E**) or elsewhere. Immunization of both strains with SCH-CI led to severe disease and this was reflected in intense inflammatory lesion in meninges (**B** and **F**), white matter (**C** and **G**), as well as some lesions in the gray matter (**D** and **H**). These tissues were also stained for iNOS and **arrows** indicate positive-staining areas. Magnification, $\times 25$.

Figure 5. Sections serial to those in Figure 4 stained immunohistochemically for ED-1⁺ cells (macrophages/microglia). It should be noted that although the quantitative comparison of lesions between the Lewis and BN when immunized with SCH-CI were very similar (Figure 4), there is a dramatic qualitative difference in lesions between the two rat strains with ED-1⁺ cells making up the majority of the inflammatory cell population in the BN (**F** to **H**) but not in the Lewis (**B** to **D**). Also note little iNOS expression (Figure 4) on this extensive macrophage/microglia infiltration. Magnification, $\times 25$.

We examined immunohistochemically, sections serial to those shown in Figure 4 for various phenotypic cell markers and the striking result observed was with respect to ED-1⁺ cells. As shown in Figure 5, a large proportion of the inflammatory cells seen in the meninges (Figure 5F), white matter (Figure 5G), and gray matter (Figure 5H) of the BN rats are ED-1⁺ ie, macrophages/microglia. The inflammatory lesions in the Lewis rats, however, showed only minimal involvement of such cells (Figure 5, B to D). We made no attempt to quantify the proportion of ED-1⁺ cells but the areas shown are representative of random fields taken from three sections at three different levels of the spinal cord. Quite interestingly, despite this intense macrophage/microglial infiltration and activation in the BN rat there are few cells expressing iNOS. Figure 4 has

also been stained immunohistochemically for iNOS; note the reddish brown staining indicated by arrows.

Cytokine Gene Expression in Draining Lymph Nodes and Spinal Cord of Lewis and BN Rats Immunized with SCH-CFA or SCH-Carbonyl Iron

In an attempt to determine whether the BN rat was responding to immunization with SCH-carbonyl iron by developing a Th1 or Th2 response, we compared cytokine mRNA expression in draining lymph node cells from Lewis and BN rats after immunization with either SCH-

Table 4. Lymph Node Cytokine Gene Expression

Rat strain	Cytokine				
	IL2	IFN γ	TNF α	IL4	IL10
Lewis					
SCH-CFA	0.86 $\pm$ 0.07	0.84 $\pm$ 0.03	0.45 $\pm$ 0.15	0.61 $\pm$ 0.04	0.56 $\pm$ 0.05
SCH-CI	0.84 $\pm$ 0.03	0.47 $\pm$ 0.04*	0.47 $\pm$ 0.05	0.50 $\pm$ 0.06	0.60 $\pm$ 0.03
BN					
SCH-CFA	0.69 $\pm$ 0.08	0.84 $\pm$ 0.15	0.65 $\pm$ 0.07	0.68 $\pm$ 0.08	0.78 $\pm$ 0.03*
SCH-CI	0.68 $\pm$ 0.08	0.43 $\pm$ 0.05*	0.54 $\pm$ 0.08	0.67 $\pm$ 0.13	0.51 $\pm$ 0.06

The popliteal lymph nodes of two Lewis rats and four BN rats were taken on day 7 after immunisation. Relative amounts of cytokines compared to β -actin. *, difference of over 40% in comparison with the value for the other adjuvant.

CFA or SCH-carbonyl iron. At day 7 after immunization with either adjuvant, the draining lymph nodes of both Lewis and BN rats showed message for the five cytokines studied: IL2, IFN γ , TNF α , IL4, and IL10 (Table 4). The Lewis rat showed higher expression of IL2 than the BN rat irrespective of the adjuvant used. Also with respect to the type of adjuvant, there was lower expression of IFN γ after immunization with SCH-carbonyl iron in both rat strains. The other difference in the cytokine profile in the draining lymph nodes was that the BN expressed higher levels of IL10 after immunization with SCH-CFA than after SCH-CI. The same differences seen at day 7 were also observed at day 12 (not shown). In the lumbar spinal cord of animals with clinical EAE at day 14, Lewis rats immunized with SCH-CFA expressed TNF α , IFN γ , IL-4, and IL-10 whereas the BN rats, taken at the same time yet having no disease when immunized with SCH-CFA, had only background levels of these cytokines. Both Lewis and BN rats immunized with SCH-CI have the same profile as the Lewis with CFA except for a very low expression of IFN γ (data not shown).

Discussion

Here, we have demonstrated that the resistance of the BN rat to EAE may be attributed, at least in part, to its ability to make high levels of NO following stimulation. *In vitro*, splenocytes from BN rats make more NO following IFN γ stimulation than do PVG splenocytes and the PVG splenocytes make more than Lewis splenocytes. This observation is in agreement with previous data for splenocytes incubated with ConA or IFN γ .^{28–30} *In vivo*, following immunization with neuroantigen and CFA, the BN and PVG rats show equal amounts of serum NO, both of which are three to four times higher than the Lewis, and the two former are resistant to EAE induction. We have previously shown¹¹ that the PVG rat could be converted to high susceptibility by inhibiting NO production with the NOS inhibitor L-NMA, however, such treatment of the BN rat failed to reduce serum NO levels or render the rats EAE susceptible. It now appears that the BN rat may in fact produce significantly more NO than the PVG but the RNI are rapidly excreted in the urine. This is most likely a homeostatic mechanism to prevent the build up of toxic concentrations of nitrate and nitrite in the blood. This apparent increased production may account for our inability to pharmacologically reduce RNI levels in the BN rat sufficiently to convert them to susceptibility. Where the high levels of NO are produced is not known but we have shown here that, following immunization with CFA, the draining lymph nodes, spleen, and induced peritoneal exudate cells all express high levels of iNOS which are not present following CI immunization. A previous study from this lab showed that the increased serum NO level in the PVG compared to the Lewis rat when immunized with MBP-CFA was most likely produced by the spleen as splenectomy of the PVG rats lowered their NO levels following immunization and converted these relatively EAE-resistant rats to high susceptibility.¹⁴ The increased

NO production by BN spleen cells *in vitro* suggests the spleen may be over-producing NO *in vivo* as well.

Here we used an adjuvant, carbonyl iron, first described almost 30 years ago by Levine and Sowinski²⁰ to induce EAE in the BN rat. We demonstrate that SCH-CI immunization does not induce an increase in NO production as SCH-CFA does and the BN rats subsequently develop severe disease. If CFA is administered simultaneously with SCH-CI, serum RNI levels increase significantly and the rats again become resistant to development of disease. Thus, lowering NO levels enhances disease while replacing increased NO levels again restores resistance, strongly suggesting that NO contributes significantly to the resistance of the BN rat to EAE.

A number of mechanisms have previously been proposed to explain the resistance of the BN rat to EAE. First, of course, is the demonstration that susceptibility or resistance is partially controlled by genes encoded by a region within the MHC locus. However, the fact that congenic strains with the Lewis MHC expressed on the BN background are still resistant to EAE indicates clearly that genes outside the MHC also play a significant role.^{18,19,21,31,32} Steroids have been examined as possible regulating molecules and indeed the BN rat has been shown to have increased basal levels of corticosterone compared to Lewis and other EAE susceptible strains.³³ However, treatment of BN rats with the steroid antagonist RU486 (Mifepristone) failed to influence the resistance to EAE. Similarly, adrenalectomy before sensitization did not allow the development of clinical EAE.²³ Furthermore, even though DA and BN rats both produce significantly more ACTH and corticosterone than the Lewis following environmental stress, the DA are highly susceptible to EAE while the BN rats remain resistant.³⁴ The consensus would seem to be, therefore, that the hypothalamic-pituitary-adrenocortical axis characteristics of a rat strain are not a predictor of disease susceptibility.

Other studies suggest that the Th1/Th2 bias of the CD4⁺ T cell population in the various strains determines the type of autoimmunity that develops. Thus the Lewis mounts a Th1 response and is susceptible to EAE and autoimmune uveoretinitis, both Th1 mediated, while the BN is susceptible to mercuric chloride-induced glomerulopathy³⁵ and gold salts autoimmunity,³⁶ both Th2 responses. Cautain et al²⁵ suggested that the natural resistance of the BN to EAE is associated with a defect in the ability to mount a Th1 response. Thus, BN lymph nodes produce more IL-4 than Lewis lymph nodes and the serum of BN rats have high amounts of TGF β following MBP immunization. Neutralization of TGF β rendered BN rats susceptible to EAE. These investigators examined the origin of the TGF β and excluded CD8 T cell involvement in its production. Interestingly, in another paper by the same group²⁶ they report that the CD8 compartment of the BN rat plays a dominant role in their inability to mount a Th1 type response. In this latter *in vitro* study it was shown that the CD8 compartment of the BN rat was only one-third that of the Lewis rat and produced only 5% of the IFN γ produced by Lewis rats. This study also reported that even though BN T cells proliferate less than Lewis T cells to Con A, (Lew X BN) F1 T cells

proliferated to Con A stimulation equally as well with either BN or Lewis splenocytes as APC, suggesting that the defect is in the T cell compartment and not the APC. Also in an APC-free stimulation culture using anti-TCR and anti-CD28, BN T cells proliferated slightly less than Lewis cells. This effect was, however, quite dose dependent in that when a lower dose of anti-CD28 was used proliferation of the two cell types was the same.

In the present work our attempt to determine the Th phenotype of the BN rat with EAE induced with SCH-CI was inconclusive. We did not do quantitative PCR and looked simply for large differences. What can be said is that, in general, the BN rat does make more IL-10 than the Lewis rat, which is in agreement with the work of others.³⁷ Also in both the lymph nodes and spinal cord of the BN rat immunized with CI there was less of the Th1 cytokine IFN γ . This does not, however, necessarily suggest that CI is inducing a Th2 disease. Work from our lab^{38,39} and others⁴⁰ has demonstrated in the mouse that even though EAE is a Th1-mediated disease, IFN γ is not essential for its expression and in fact appears to be essential for recovery from disease. If the mouse work can be extrapolated to the rat then the fact that CI-immunized BN rats responded with IL-2 and TNF α production suggests a Th1 response.

Whether SCH-CI-induced EAE is a Th1 or Th2 response may in fact be a moot point. We do not doubt that the BN and the Lewis rat preferentially produce different cytokines on immunization with the same antigen. What we suggest, however, is that this Th bias may be due to a differential ability to produce NO. NO has been shown to influence immune reactivity in a number of ways. NO inhibits lymphocyte proliferation probably by preventing the activation of Janus kinases,⁴¹ molecules critical in cytokine receptor signal transduction.⁴² Other studies have described a NO-induced bias of cytokine gene expression in which NO preferentially down-regulates Th1-type cytokines specifically while up-regulating Th2-associated molecules, resulting in a Th2 bias.⁴³ Such bias would prevent expansion of Th1 cells essential for EAE pathology. A similar regulatory effect might be exerted by NO at the level of antigen presentation as it has been shown that NO induces transcription of the IL-12 p40 gene in macrophages but not the p35 gene. Because the p40 homodimer acts as an antagonist for IL-12,⁴⁴ this might lead to less Th1 reactivity in the presence of NO.

Yet another possible limiting effect of NO on immune reactivity is its ability to inhibit T cell migration. We have shown, at least *in vitro*, that NO inhibits migration of encephalitogenic T cells through CNS endothelial monolayers in a dose-dependent manner.⁴⁵ This migration inhibition is thought to be due to NO's ability to polarize T lymphocytes resulting in a morphology suggestive of migrating cells, ie, ones with a leading edge and trailing uropod. We suggested that these NO-induced morphological changes result in T cells with predefined migratory directionality, thus limiting the options these cells have to respond to other migratory signals. The genetic loci outside the MHC described as contributing to susceptibility to autoimmune disease may contain elements regulating iNOS gene expression.

Complete Freund's adjuvant can be used in the BN rat to induce EAE if myelin oligodendroglial glycoprotein (MOG) is used as antigen.⁴⁶ MOG-induced EAE requires both T cells and antibody for its full expression and the resultant CNS pathology in the BN rat is characterized by the deposition of C9 and IgG and a cellular infiltrate comprised of large numbers of neutrophils as well as eosinophils. Interestingly, in the MOG model, there were fewer macrophages in the infiltrates of the BN rat than the Lewis rat. These investigators concluded that the disease in the BN rat induced with MOG was independent of a substantial Th2 T cell response and was mediated by Th1 CD4⁺ T cells with clinical expression dependent on anti-MOG antibody.

The pathology seen in the BN rat immunized with carbonyl iron as adjuvant is of considerable interest. Both the Lewis and the BN rats immunized with CI had extensive inflammatory lesions in the meninges, white and gray matter which, based on H&E staining, were of equal intensity as well as similar distribution. The lesions were, however, qualitatively different between the strains. Thus, lesions in the BN rat had extensive involvement of ED-1⁺ cells whereas there was considerably less involvement of these cells in lesions seen in the Lewis rat. This was true in all three of each type of rat examined. The reason for this difference is not known. It should be remembered that the antigen used was whole spinal cord homogenate and not a defined antigen. The different strains of rat may be responding to different neuroantigens within the homogenate leading to different cellular responses. Conversely, the rats may be responding to the same antigen but with a different cellular response. ED-1 defines both peripheral macrophages and resident activated microglia and therefore either of these cells may be selectively up-regulated by the BN and not the Lewis. We are currently investigating whether the ED-1⁺ cells in the BN are predominately macrophages or microglia.

This difference in pathology between strains of rats in this study certainly has parallels with the human disease multiple sclerosis (MS). MS is extremely heterogeneous in its clinical presentation, response to therapy, radiological findings, structural pathology, and pathogenesis. There is no way of determining which patients will develop which type of disease. One form of disease is acute MS, or Marburg's variant. It is a rare event but runs an exceptionally severe course, which typically ends in death within a year of diagnosis. Interestingly the pathology of Marburg's is characterized by extensive macrophage infiltration rather than the more usual lymphocytic response seen in other forms of MS. Marburg's may also be associated with an altered myelin basic protein structure and the deposition of immunoglobulins.^{47,48}

Finally, another important question raised by this work is what is the mechanism of adjuvanticity of carbonyl iron? It was interesting to note that the footpads of CI-immunized rats showed virtually no inflammation or swelling when compared to immunization with CFA. Also the draining lymph nodes of the CI immunized rats had substantially fewer macrophages, as determined by ED-1 staining, yet the BN rat developed severe EAE with extensive macrophage infiltration into the CNS. The

mechanism(s) by which carbonyl iron exerts its adjuvant activity is under investigation.

Acknowledgments

We thank Anne Prins for the excellent histology work, Dr. Giang Tran for the help with the PCR for IL4 and IL10, David Linares for the TNF α primers, and Mark Fenning, Karen Edwards, and Julie Macklin from the photolab of JCSMR.

References

- Marlatta MA: Nitric oxide: biosynthesis and biological significance. *Trends Biochem Sci* 1989, 14:488–492
- Moncada S, Palmer RM, Higgs EA: Nitric oxide: physiology, pathophysiology, and pharmacology. *Pharmacol Rev* 1991, 43:109–142
- Lowenstein CJ, Snyder SH: Nitric oxide, a novel biologic messenger. *Cell* 1992, 70:705–707
- Nathan CF, Hibbs JB: Role of nitric oxide synthesis in macrophage antimicrobial activity. *Curr Opin Immunol* 1991, 3:65–70
- Willenborg DO, Staykova MA, Cowden WB: Our shifting understanding of the role of nitric oxide in autoimmune encephalomyelitis: a review. *J Neuroimmunol* 1999, 100:21–35
- Sherman MP, Griscavage JM, Ignarro LJ: Nitric oxide-mediated neuronal injury in multiple sclerosis. *Med Hypotheses* 1992, 39:143–146
- Lin RF, Lin TS, Tilton RG, Cross AH: Nitric oxide localized to spinal cords of mice with experimental allergic encephalomyelitis: an electron paramagnetic resonance study. *J Exp Med* 1993, 178:643–648
- Koprowski H, Zheng YM, Heber-Katz E, Fraser N, Rorke L, Fu ZF, Hanlon C, Dietzschold B: In vivo expression of inducible nitric oxide synthase in experimentally induced neurologic diseases. *Proc Natl Acad Sci USA* 1993, 90:3024–3027
- Cross AH, Misko TP, Lin RF, Hickey WF, Trotter JL, Tilton RG: Aminoguanidine, an inhibitor of inducible nitric oxide synthase, ameliorates experimental autoimmune encephalomyelitis in SJL mice. *J Clin Invest* 1994, 93:2684–2690
- MacMicking JD, Willenborg DO, Weidemann MJ, Rockett KA, Cowden WB: Elevated secretion of reactive nitrogen and oxygen intermediates by inflammatory leukocytes in hyperacute experimental autoimmune encephalomyelitis: enhancement by the soluble products of encephalitogenic T cells. *J Exp Med* 1992, 176:303–307
- Cowden WB, Cullen FA, Staykova MA, Willenborg DO: Nitric oxide is a potential down-regulating molecule in autoimmune disease: inhibition of nitric oxide production renders PVG rats highly susceptible to EAE. *J Neuroimmunol* 1998, 88:1–8
- Ruuls SR, Van Der Linden S, Sontrop K, Huitinga I, Dijkstra CD: Aggravation of experimental allergic encephalomyelitis (EAE) by administration of nitric oxide (NO) synthase inhibitors. *Clin Exp Immunol* 1996, 103:467–474
- Zielasek J, Tausch M, Toyka KV, Hartung HP: Production of nitrite by neonatal rat microglial cells/brain macrophages. *Cell Immunol* 1992, 141:111–120
- Staykova MA, Cowden W, Willenborg DO: Macrophages and nitric oxide as the possible cellular and molecular basis for strain and gender differences in susceptibility to autoimmune central nervous system inflammation. *Immunol Cell Biol* 2002, 80:188–197
- O'Brien NC, Charlton B, Cowden WB, Willenborg DO: Nitric oxide plays a critical role in the recovery of Lewis rats from experimental autoimmune encephalomyelitis and the maintenance of resistance to reinduction. *J Immunol* 1999, 163:6841–6847
- O'Brien NC, Charlton B, Cowden WB, Willenborg DO: Inhibition of nitric oxide synthase initiates relapsing remitting experimental autoimmune encephalomyelitis in rats, yet nitric oxide appears to be essential for clinical expression of disease. *J Immunol* 2001, 167:5904–5912
- Willenborg DO: Experimental allergic encephalomyelitis in the Lewis rat: studies on the mechanism of recovery from disease and acquired resistance to reinduction. *J Immunol* 1979, 123:1145–1150
- Williams RM, Moore MJ: Linkage of susceptibility to experimental allergic encephalomyelitis to the major histocompatibility locus in the rat. *J Exp Med* 1973, 138:775–783
- Gasser DL, Palm J, Gonatas NK: Genetic control of susceptibility to experimental allergic encephalomyelitis and the Ag-B locus of rats. *J Immunol* 1975, 115:431–433
- Levine S, Sowinski R: Allergic encephalomyelitis in the reputedly resistant Brown Norway strain of rats. *J Immunol* 1975, 114:597–601
- Happ MP, Wettstein P, Dietzschold B, Heber-Katz E: Genetic control of the development of experimental allergic encephalomyelitis in rats: separation of MHC and non-MHC gene effects. *J Immunol* 1988, 141:1489–1494
- Issazadeh S, Kjellen P, Olsson T, Mustafa M, Holmdahl R: Major histocompatibility complex-controlled protective influences on experimental autoimmune encephalomyelitis are peptide specific. *Eur J Immunol* 1997, 27:1584–1587
- Peers SH, Duncan GS, Flower RJ, Bolton C: Endogenous corticosteroids modulate lymphoproliferation and susceptibility to experimental allergic encephalomyelitis in the Brown Norway rat. *Int Arch Allergy Immunol* 1995, 106:20–24
- Cautain B, Damoiseaux J, Bernard I, Fournie E, van Breda Vriesman P, Druet P, Saoudi A: Non-MHC determined resistance of Brown-Norway rats to develop experimental allergic encephalomyelitis is mediated by the endogenous production of transforming growth factor-beta. *Transplant Proc* 1999, 31:1602–1603
- Cautain B, Damoiseaux J, Bernard I, van Straaten H, van Breda Vriesman P, Boneu B, Druet P, Saoudi A: Essential role of TGF-beta in the natural resistance to experimental allergic encephalomyelitis in rats. *Eur J Immunol* 2001, 31:1132–1140
- Cautain B, Damoiseaux J, Bernard I, Xystrakis E, Fournie E, van Breda Vriesman P, Druet P, Saoudi A: The CD8 T cell compartment plays a dominant role in the deficiency of Brown-Norway rats to mount a proper type 1 immune response. *J Immunol* 2002, 168:162–170
- Eylar EH, Jackson JJ, Rothenberg B, Brostoff SW: Suppression of the immune response: reversal of the disease state with antigen in allergic encephalomyelitis. *Nature* 1972, 236:74–76
- Fu Y, Blankenhorn EP: Nitric oxide-induced anti-mitogenic effects in high and low responder rat strains. *J Immunol* 1992, 148:2217–2222
- Ozaki A, Fukushima A, Fukata K, Sakamoto S, Taniguchi T, Hashiguchi M, Ueno H: Genetic background determines sensitivity to the inhibitory function of NO on T cell proliferation and the amounts of NO production mediated through IFN-gamma. *Microbiol Immunol* 2002, 46:555–563
- van der Meide PH, de Labie MC, Botman CA, Aten J, Weening JJ: Nitric oxide suppresses IFN-gamma production in the spleen of mercuric chloride-exposed Brown Norway rats. *Cell Immunol* 1995, 161:195–206
- Gasser DL, Newlin CM, Palm J, Gonatas NK: Genetic control of susceptibility to experimental allergic encephalomyelitis in rats. *Science* 1973, 181:872–873
- Moore MJ, Singer DE, Williams RM: Linkage of severity of experimental allergic encephalomyelitis to the rat major histocompatibility locus. *J Immunol* 1980, 124:1815–1820
- Villas PA, Dronsfield MJ, Blankenhorn EP: Experimental allergic encephalomyelitis and corticosterone studies in resistant and susceptible rat strains. *Clin Immunol Immunopathol* 1991, 61:29–40
- Steffertl A, Linington C, Holsboer F, Reul JM: Susceptibility and resistance to experimental allergic encephalomyelitis: relationship with hypothalamic-pituitary-adrenocortical axis responsiveness in the rat. *Endocrinology* 1999, 140:4932–4938
- Fournie GJ, Cautain B, Xystrakis E, Damoiseaux J, Mas M, Lagrange D, Bernard I, Subra JF, Pelletier L, Druet P, Saoudi A: Cellular and genetic factors involved in the difference between Brown Norway and Lewis rats to develop respectively type-2 and type-1 immune-mediated diseases. *Immunol Rev* 2001, 184:145–160
- Savignac M, Badou A, Delmas C, Subra JF, De Cramer S, Paulet P, Cassar G, Druet P, Saoudi A, Pelletier L: Gold is a T cell polyclonal activator in BN and LEW rats but favors IL-4 expression only in autoimmune prone BN rats. *Eur J Immunol* 2001, 31:2266–2276
- Vandebriel RJ, Meredith C, Scott MP, van Dijk M, van Loveren H: Interleukin-10 is an unequivocal Th2 parameter in the rat, whereas interleukin-4 is not. *Scand J Immunol* 2000, 52:519–524
- Willenborg DO, Fordham S, Bernard CC, Cowden WB, Ramshaw IA: IFN-gamma plays a critical down-regulatory role in the induction and

- effector phase of myelin oligodendrocyte glycoprotein-induced autoimmune encephalomyelitis. *J Immunol* 1996, 157:3223–3227
39. Willenborg DO, Fordham SA, Staykova MA, Ramshaw IA, Cowden WB: IFN- γ is critical to the control of murine autoimmune encephalomyelitis and regulates both in the periphery and in the target tissue: a possible role for nitric oxide. *J Immunol* 1999, 163:5278–5286
40. Krakowski M, Owens T: Interferon- γ confers resistance to experimental allergic encephalomyelitis. *Eur J Immunol* 1996, 26:1641–1646
41. Duhe RJ, Evans GA, Erwin RA, Kirken RA, Cox GW, Farrar WL: Nitric oxide and thiol redox regulation of Janus kinase activity. *Proc Natl Acad Sci USA* 1998, 95:126–131
42. Ihle JN: The Janus kinase family and signaling through members of the cytokine receptor superfamily. *Proc Soc Exp Biol Med* 1994, 206:268–272
43. Taylor-Robinson AW: Inhibition of IL-2 production by nitric oxide: a novel self-regulatory mechanism for Th1 cell proliferation. *Immunol Cell Biol* 1997, 75:167–175
44. Mattner F, Fischer S, Guckes S, Jin S, Kaulen H, Schmitt E, Rude E, Germann T: The interleukin-12 subunit p40 specifically inhibits effects of the interleukin-12 heterodimer. *Eur J Immunol* 1993, 23:2202–2208
45. Staykova MA, Berven LA, Cowden WB, Willenborg DO, Crouch MF: Nitric oxide induces polarization of actin in encephalitogenic T cells and inhibits their in vitro trans-endothelial migration in a p70S6 kinase-independent manner. *FASEB J* 2003, 17:1337–1339
46. Steffler A, Brehm U, Storch M, Lambracht-Washington D, Bourquin C, Wonigeit K, Lassmann H, Linington C: Myelin oligodendrocyte glycoprotein induces experimental autoimmune encephalomyelitis in the “resistant” Brown Norway rat: disease susceptibility is determined by MHC and MHC-linked effects on the B cell response. *J Immunol* 1999, 163:40–49
47. Wood DD, Bilbao JM, O’Connors P, Moscarello MA: Acute multiple sclerosis (Marburg type) is associated with developmentally immature myelin basic protein. *Ann Neurol* 1996, 40:18–24
48. Lucchinetti C, Bruck W, Parisi J, Scheithauer B, Rodriguez M, Lassmann H: Heterogeneity of multiple sclerosis lesions: implications for the pathogenesis of demyelination. *Ann Neurol* 2000, 47:707–717