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Substance P Augments Borrelia burgdorferi-Induced Prostaglandin E₂ Production by Murine Microglia

Amy Rasley,* Ian Marriott,† Craig R. Halberstadt,‡ Kenneth L. Bost,* and Juan Anguita*

Substance P is a ubiquitous CNS neuropeptide and has recently been demonstrated to augment immune cell function during inflammatory events. Central to the ability of substance P to modulate immune cell function is the interaction of substance P with the substance P neurokinin-1 receptor expressed by a variety of immune cells, including microglia. CNS involvement during Lyme disease can occur when Borrelia burgdorferi, the causative agent of Lyme disease, gains access to the CNS. In the present study, we demonstrate that substance P augments B. burgdorferi-induced expression of mRNA encoding COX-2 and subsequent secretion of PGE₂, by cultured, murine microglia. Furthermore, this effect is associated with the ability of substance P to enhance B. burgdorferi-induced NF-κB activation, as demonstrated by increased nuclear localization of the p65 (RelA) subunit of NF-κB in these cells. Interestingly, we demonstrate that substance P augments B. burgdorferi-induced expression of mRNA encoding two PGE₂ receptors, E-prostanoid receptor subtypes 2 and 4, as well as each receptor protein. In addition, these effects are mediated via interactions between substance P and its high affinity receptor, as evidenced by the absence of augmented PGE₂ synthesis in the presence of a specific neurokinin-1 receptor antagonist or in cells genetically deficient in the expression of these receptors. Taken together, the present demonstration that substance P can exacerbate B. burgdorferi-induced inflammatory responses in microglia in vitro may indicate a role for this neuropeptide in the development of CNS inflammation observed during human neuroborreliosis. The Journal of Immunology, 2004, 172: 5707–5713.

A compelling body of evidence has accumulated to indicate that the neuropeptide, substance P (SP), plays an important role in augmenting inflammatory immune responses (1). This is evidenced by diminished inflammatory responses in studies in which SP/SP receptor interactions are inhibited in vivo (2–4), or in studies using mice genetically devoid of SP-SP receptor interactions (5–7). Central to the ability of SP to augment inflammation is the finding that this neuropeptide can modulate the function of myeloid cells, such as macrophages and dendritic cells, via SP-specific neurokinin (NK-1) (3) receptors (8–11).

Microglia are resident immune cells of the CNS (3) and, like macrophages and dendritic cells, are of myeloid lineage. Consequently, these cells are likely to play an important role in either development of protective immune responses or progression of damaging inflammation during CNS disease states (12–14). Microglia respond to traumatic injury, or the presence of infectious organisms, by migrating to the site of injury where they proliferate. Microglia become activated at the site of challenge and assume many of the immune effector functions typically associated with macrophages. For example, they are known to be facultative phagocytes, and express Ag-presenting MHC class II molecules (15). Importantly, microglia can also be induced to produce key proinflammatory molecules such as TNF-α and IL-6 (16, 17). As such, these cells are ideally suited to detect and respond to pathogens that infiltrate the CNS. Recent studies by our laboratory have demonstrated the presence of functional NK-1R on murine microglia (18, 19). However, the ability of SP to modulate microglial responses to microbial challenge has not been investigated.

Lyme disease is a multisystemic infection that involves a variety of organ systems, including the CNS (20, 21). The invasion of the CNS by Borrelia burgdorferi, the causative agent of Lyme disease, can lead to damaging inflammation associated with marked levels of proinflammatory molecules such as IL-6 and TNF-α (22, 23). The majority of clinical symptoms of Lyme disease are due to the localized presence of B. burgdorferi in various tissues (24). Such infections have been associated with the up-regulation of the important inflammatory enzyme, cyclooxygenase-2 (COX-2) (25).

In the present study, we have investigated the ability of the neuropeptide SP to augment B. burgdorferi-induced microglial responses. We demonstrate that SP markedly augments B. burgdorferi-induced expression of mRNA encoding the key inflammatory enzyme COX-2 by cultured murine microglia. Importantly, this increase in the expression of mRNA encoding COX-2 translated into increased secretion of the prostanoid, PGE₂. This induction is associated with an augmented increase in the nuclear localization of the key transcriptional activator, NF-κB. In addition, we demonstrate that SP synergizes with B. burgdorferi to augment levels of expression of mRNA encoding the PGE₂ receptors, E-prostanoid receptor subtypes 2 and 4 (EP2 and EP4), as well as enhanced receptor protein expression.

Taken together, the ability of SP to augment B. burgdorferi-induced microglial responses in vitro may provide a possible explanation whereby this bacterium can elicit inflammatory responses within the CNS during neuroborreliosis.

Materials and Methods

SP receptor-deficient mice

SP receptor-deficient mice, bred for >10 generations onto a C57BL/6 background, were derived at the University of Iowa Medical Center (Iowa...
City, IA) (26). These mice were originally derived from induced mutations made by insertion of the lacZ gene into exon 1 of the SP receptor (5). SP receptor-deficient mice were routinely screened by PCR to confirm disruption of the SP receptor, as previously described (5, 27), using the positive and negative strand primers CCAACACTCAAGACTCTCG and GCCAGACCTAGTGTATGAGTCTGAGTATG for SP receptor deficiency, respectively.

**Isolation of primary murine microglia**

Murine neonatal brain microglia were isolated, as described previously, by our laboratory (18, 19, 28). Briefly, six to eight neonatal BALB/c or C57BL/6 (NK-1r−/−) mouse brains per preparation were dissected free of meninges and large blood vessels, and minced with sterile surgical scissors. Then, the mince was forced through a wire screen and brieﬂy incubated with 0.01% trypsin-0.005% EDTA for 5 min. The cell suspension was then washed, and this mixed glial culture was maintained in RPMI 1640 containing 10% FBS and gentamicin for 2 wk. A microglia culture was obtained from this mixed glial culture by shaking ﬂasks for 1 h at 200 rpm in an orbital shaker (New Brunswick Scientiﬁc, Edison, NJ) and allowing the transferred dislodged cells to adhere to new tissue culture vessels for 1 h. The medium was then removed, and fresh RPMI 1640 with 10% FBS and 20% conditioned medium from LADMAC cells (ATCC number CRL-2430) was added to serve as a source of CSF-1 to maintain microglia cultures for 1 wk, as described previously (29). Cells isolated in this manner were previously demonstrated to be >95% authentic microglia due to their characteristic morphology and the presence of the microglia cell surface markers CD11b and F4/80, as determined by FACS analysis (19).

**Preparation of B. burgdorferi Ags**

A clonal low passage of B. burgdorferi strain N40 was used throughout these studies (30). Spirochetes were grown in Barbour-Stoenner-Kelly II medium (31). Cells were centrifuged at 10,000 × g and washed three times with PBS. After the ﬁnal wash, bacteria were resuspended in PBS and pulsed three times with a cell sonicator for 20 s at 25-s intervals. B. burgdorferi Ags isolated in this manner have been previously demonstrated to be free of detectable levels of LPS (25). Isolated microglia were exposed to 1 µg/ml B. burgdorferi cell lysate, as indicated.

**Isolation of RNA and semiquantitative RT-PCR**

Total RNA was isolated from microglia using TRIzol reagent (Life Technologies, Gaithersburg, MD), as described previously (18, 19, 28). PCR was performed to determine the expression of COX-1, COX-2, EP2R, and EP4R. PCR was performed on 5% of the reverse-transcribed cDNA product to quantify expression of the mRNAs encoding COX-2, COX-1, EP2R, EP4R, and G3PDH using 95°C denaturation, 60°C annealing, and 72°C extension temperatures (Robocycler 40; Stratagene, La Jolla, CA) with the manufacturer’s instructions (Molecular Probes), and analyzed by confocal microscopy (Olympus, Melville, NY) using a ×60 objective.

**Densiometric analyses**

Densiometric analyses of RT-PCR products were performed using NIH Image (obtained from the National Institutes of Health website: http://tis-b.info.nih.gov/nih-image). Each image was imported into NIH Image by Adobe Photoshop (Adobe Systems, San Jose, CA), a gel-ploting macro was used to outline the bands, and the intensity was calculated on the uncalibrated OD setting.

**Results**

SP augments B. burgdorferi-induced COX-2 mRNA expression by microglia

To begin to determine whether SP is capable of modulating microglial responses, we investigated the effect of SP and B. burgdorferi stimulation on levels of expression of mRNA encoding the enzyme, COX-2, in primary murine microglia. Microglia were cultured in the presence or absence of B. burgdorferi Ags (Bb) at a dose below that previously shown to elicit maximal inﬂammatory responses by cultured microglia (1 µg/ml) (18), and with or without SP (1 nM) at a dose that has previously been demonstrated to activate the transcription factor NF-κB in cultured microglia (19). At the indicated times posttreatment, RNA was isolated, and semi-quantitative RT-PCR was performed for the presence of COX-2 mRNA expression. As shown in Fig. 1A, stimulation with Bb or SP alone failed to induce the expression of mRNA encoding COX-2.
after 4 h, although these agents did elicit modest elevations in mRNA encoding COX-2 after 8 h (1.3- and 2.2-fold increases, as measured by densitometric analysis, respectively) (Fig. 1A). Intriguingly, simultaneous exposure of microglia to Bb and SP resulted in a marked increase in the levels of expression of mRNA encoding COX-2 as rapidly as 4 h posttreatment (46-fold increase, as measured by densitometric analysis) (Fig. 1A). This effect of SP on Bb-induced COX-2 mRNA expression was sustained after 8 h (12.5-fold increase, as measured by densitometric analysis), but abated after 12 h posttreatment (Fig. 1A).

To confirm the specificity of the SP effects on COX-2 mRNA expression, we used a specific nonpeptide, NK-1R antagonist, L-703,606 (Sigma-Aldrich, St. Louis, MO). Microglia were either treated or untreated with L-703,606 (10 nM) for 15 min before exposure of the cells to Bb and SP. As shown in Fig. 1A, L-703,606 attenuates the effect of SP on B. burgdorferi-induced COX-2 mRNA expression seen at 4 and 8 h posttreatment (50 and 52% inhibition, as measured by densitometric analysis, respectively). To ensure that differences in mRNA levels could not be attributed to differences in input RNA, or efficiencies of reverse transcription, RT-PCR amplification of the housekeeping gene, G3PDH, was performed for each sample (Fig. 1A).

To further confirm the NK-1R-mediated ability of SP to augment the levels of expression of mRNA encoding COX-2, we isolated microglia from mice genetically deficient in the expression of the SP receptor (NK-1R−/-mice). NK-1R−/- microglia were cultured in the presence or absence of Bb Ags (1 μg/ml), SP (1 nM), or both stimuli together. At the indicated times posttreatment, RNA was isolated, and semiquantitative RT-PCR was performed for the presence of COX-2 mRNA expression. As shown in Fig. 1B, stimulation with Bb alone failed to elicit increased levels of expression of mRNA encoding COX-2, consistent with experiments using wild-type microglia (Fig. 1A). As expected, the lack of SP/NK-1R receptor interactions in these cells resulted in a loss of synergistic increases in levels of expression of mRNA encoding COX-2 in the presence of Bb and SP (Fig. 1B), confirming a NK-1R-mediated ability of SP to augment B. burgdorferi-induced microglial responses. Taken together, these results demonstrate the ability of the neuropeptide, SP, to enhance and specifically augment B. burgdorferi-induced microglial responses in vitro.

Levels of mRNA encoding COX-1 are constitutive and are unaffected by B. burgdorferi and SP stimulation

COX-1 has been shown to be constitutively expressed in a variety of tissues, including the CNS. To determine whether the ability of SP to augment B. burgdorferi-induced COX expression is restricted to the inducible form of the enzyme (COX-2), we investigated the effect of SP and B. burgdorferi stimulation on levels of expression of mRNA encoding COX-1 in primary murine microglia. Microglia were cultured in the presence or absence of B. burgdorferi Ags (Bb) (1 μg/ml), SP (1 nM), or both stimuli together. At the indicated times posttreatment, RNA was isolated, and semiquantitative RT-PCR was performed for the presence of COX-1 mRNA expression. As shown in Fig. 2, levels of mRNA expression encoding COX-1 are not significantly altered from constitutive expression, in both wild-type microglia and microglia isolated from NK-1R−/- mice (Fig. 2). Taken together, these results demonstrate that, in contrast to COX-2, levels of expression of COX-1 mRNA are independent of Bb and SP treatment.
microglial culture supernatants. Culture supernatants of untreated primary microglia or microglia treated with *B. burgdorferi* (Bb) alone (1 μg/ml), SP alone (1 nM), or both stimuli together were harvested 4 h posttreatment and assayed for PGE2 production. As shown in Fig. 3A, treatment of microglia with Bb or SP alone failed to elicit detectable levels of PGE2 production by these cells. Importantly, simultaneous exposure to both Bb and SP resulted in a marked increase in PGE2 secretion by microglia (Fig. 3A) compared with untreated cells or cells exposed to either stimulus alone (Fig. 3A). The effect of SP- on Bb-induced PGE2 production was dependent on SP/SP receptor interactions, as shown by its sensitivity to the presence of the specific SP receptor antagonist, L-703,606 (10 nM) (Fig. 3A), which by itself or in combination with Bb or SP had no significant effect on the production of this prostaglandin by microglia (Fig. 3A). In addition, to confirm the specificity of the effects of SP on Bb-mediated PGE2 production, similar experiments were performed using microglia derived from NK-1R-deficient mice. As shown in Fig. 3B, treatment of microglia with *B. burgdorferi* alone or SP alone failed to elicit detectable levels of PGE2 production by these cells. Importantly, exposure to both Bb and SP simultaneously had no effect on levels of PGE2 secretion by these cells after 4 h (Fig. 3B) or 8 h (data not shown), confirming the importance of SP/SP receptor interactions in augmenting *B. burgdorferi*-induced microglial responses (Fig. 3B). For comparison purposes and to ensure the functionality of the assay, culture supernatants from primary microglia were harvested 4 h after stimulation with *B. burgdorferi* (5 Bb) at a concentration previously demonstrated to maximally induce PGE2 production by cultured microglia (5 μg/ml) (18) and is shown in Fig. 3B.

**FIGURE 3.** SP augments *B. burgdorferi*-induced PGE2 production. A. Cultured primary murine microglia were untreated (−) or treated (+) with *B. burgdorferi* lysate (Bb) (1 μg/ml), SP (1 nM), NK-1R antagonist (NK-1R Antag) (10 nM), or Bb + SP + NK-1R antagonist, and culture supernatants were taken 4 h postexposure. A specific capture ELISA was performed to quantify PGE2 secretion. B. Cultured NK-1R−/− primary murine microglia were untreated (−) or exposed (+) to *B. burgdorferi* lysate (Bb) (1 μg/ml), SP (1 nM), or both stimuli together, and culture supernatants were taken 4 h postexposure. A specific capture ELISA was performed to quantify PGE2 secretion. For comparison purposes and to ensure the functionality of the assay, culture supernatants from primary microglia were harvested 4 h after stimulation with 5 μg/ml Bb whole cell lysate (5 Bb). Results are presented as the mean of three experiments ± SEM (#, denotes p < 0.05).

SP augments *B. burgdorferi*-induced activation of the transcription factor, NF-κB, in murine microglia

Given the ability of both *B. burgdorferi* and SP to elicit activation of NF-κB in murine microglia (18, 19), we hypothesized that simultaneous stimulation of cultured microglia with *B. burgdorferi* and SP would result in a marked increase in the activation of NF-κB when compared with untreated cells or cells treated with either stimulus alone.

Microglia were untreated or treated with *B. burgdorferi* (Bb) Ags (1 μg/ml), SP (1 nM), or both stimuli together for 30 min and assayed for the cellular localization of the p65 (RelA) subunit of NF-κB via confocal microscopy. As shown in Fig. 4, treatment with either Bb or SP alone failed to elicit increased levels of RelA in the nucleus when compared with untreated microglia cells (Fig. 4, A–C). In contrast, microglia cells exposed to Bb and SP simultaneously exhibited a robust increase in the expression of RelA in the nucleus (Fig. 4D). Furthermore, this increase in the levels of nuclear RelA was dependent on the actions of SP through its receptor, as evidenced by the ability of L-703,606 (10 nM) to abrogate this response (Fig. 4F), while having no effect by itself when compared with untreated cells (Fig. 4E).

To confirm the ability of SP to augment *B. burgdorferi*-induced NF-κB activation, microglial nuclear extracts were analyzed for the cellular localization of the p65 (RelA) subunit of NF-κB by Western blot analysis. As shown in Fig. 5, microglia treated simultaneously with both stimuli exhibited a dramatic increase in the level of RelA present in the nucleus when compared with either treatment alone (133 vs 45 and 37 arbitrary densitometric units, respectively) (Fig. 5). Importantly, this marked induction was attenuated in the presence of the NK-1R antagonist, L-703,606 (1 nM) (56% inhibition, as measured by densitometric analysis).

Taken in concert, these data demonstrate the ability of SP to augment *B. burgdorferi*-induced NF-κB activation in cultured microglia, as evidenced by the marked increases in nuclear translocation of the RelA subunit (Figs. 4 and 5).
FIGURE 5. SP robustly increases B. burgdorferi-induced nuclear translocation of the RelA subunit of NF-κB in murine microglia as determined by Western blot analysis. Cells were untreated (−) or treated (+) with B. burgdorferi lysate (Bb) (1 μg/ml), SP (1 nM), NK-1R antagonist (NK-1R Antag) (10 nM), or Bb + SP + NK-1R antagonist for 30 min. Nuclear extracts were harvested, and 10 μg of total protein was analyzed for the presence of RelA by Western blot analysis. These experiments were performed three times with similar results.

SP markedly increases levels of mRNA encoding the PGE₂ receptors, EP2 and EP4, as well as each receptor protein, in B. burgdorferi-challenged microglia

PGE₂ has been demonstrated to exert its effects on a variety of cell types by binding to one or a combination of four subtypes of receptor designated, EP1, EP2, EP3, and EP4. Importantly, the interaction between PGE₂ and the EP receptor subtypes 2 and 4 has previously been demonstrated to modulate macrophage effector functions (38, 39) as well as potentiate inflammatory disease progression (40). Microglia cells were untreated or exposed to B. burgdorferi (Bb) Ags (1 μg/ml), SP (1 nM), or both stimuli together. RNA was isolated 4 h later, and semiquantitative RT-PCR was performed for the presence of mRNA encoding the EP2 and EP4 receptors. As shown in Fig. 6A, neither receptor subtype was constitutively expressed by untreated murine microglia. Importantly, Bb treatment alone resulted in an increase in the levels of expression of mRNA encoding the EP2 receptor (18-fold increase, as measured by densitometric analysis), while failing to elicit detectable increases in expression of mRNA encoding the EP4 receptor (Fig. 6A). Similarly, SP treatment alone evoked increases in the levels of expression of mRNA encoding both the EP2 and EP4 receptor, compared with untreated cells (49- and 49-fold increases, as measured by densitometric analysis, respectively) (Fig. 6A).

In contrast, the combined presence of B. burgdorferi and SP elicited synergistic increases in the levels of mRNA encoding both the EP2 and EP4 receptors (194- and 96-fold increases, as measured by densitometric analysis, respectively) (Fig. 6A).

To assess whether synergistic increases in mRNA encoding the EP2 and EP4 receptors in microglia translate into increases in expression of these receptor proteins, we investigated the combined effect of Bb and SP treatment on EP2 and EP4 receptor protein levels in microglia by Western blot analysis. Cells were untreated or exposed to B. burgdorferi Ags (Bb) (1 μg/ml), SP (1 nM), or both stimuli together for 12 h. As shown in Fig. 6B, Bb treatment alone had no effect on levels of EP2 and EP4 receptor protein when compared with unstimulated cells. Similarly, SP treatment alone failed to evoke an increase in the levels of expression of EP2 and EP4 receptor protein, compared with untreated and Bb-treated cells (Fig. 6B). However, the combined presence of B. burgdorferi and SP elicited marked increases in the levels of both EP2 and EP4 receptor proteins (2-fold increases in each, as measured by densitometric analysis corrected for background intensity for each lane) (Fig. 6B). Furthermore, this effect was dependent on SP/SP receptor interactions, as shown by the ability of L-703,606 (10 nM) pretreatment to inhibit this effect (100% inhibition of the increases observed, as measured by densitometric analysis) (Fig. 6B).

Taken together, these data demonstrate that Bb and SP act in concert to augment the expression of the EP receptor subtypes, 2 and 4, by primary murine microglia.

Discussion

It is now widely accepted that the neuropeptide SP can play an important role in augmenting inflammatory responses at peripheral sites. In particular, it has been demonstrated that SP can promote the immune functions of peripheral macrophages (1). Binding of this neuropeptide to SP receptors (NK-1R) augments the production of proinflammatory monokines such as IL-1, IL-6, and TNF-α (41, 42). Furthermore, SP can induce a respiratory burst in macrophages, resulting in the production of reactive oxygen intermediates (43). Although SP by itself can promote production of proinflammatory molecules, there is also evidence that this neuropeptide can augment LPS-mediated proinflammatory cytokine production (8, 9, 44) and can inhibit the production of anti-inflammatory cytokines (45). However, much less is known about the role of this neuropeptide in the initiation and/or maintenance of inflammatory responses at the site of its ubiquitous distribution, the CNS. We have recently demonstrated the presence of authentic NK-1R on isolated cultures of murine microglia, resident myeloid cells of the CNS. Importantly, we showed the functional nature of such expression by demonstrating the ability of nanomolar concentrations of SP to elicit activation of NF-κB, a transcription factor that plays a role in inflammatory cytokine production (19). However, to date, the ability of SP to augment proinflammatory responses of microglia has not been investigated.

In the present study, we have demonstrated that SP augments prostaglandin synthesis in response to a bacterial CNS pathogen. Specifically, we show that SP augments the ability of B. burgdorferi, the causative agent of Lyme disease, to elevate expression of
mRNA encoding the key proinflammatory enzyme, COX-2. This effect translated into robust increases in the secretion of the prostanoid PGE2. Furthermore, this effect is a result of SP acting via its specific receptor, as indicated by the ability of L-703,606, a specific NK-1R antagonist, to abrogate these responses, and the absence of this effect in mice genetically deficient in the expression of NK-1R. Interestingly, SP increases the expression of the two prostanoid receptors associated with inflammatory effects, EP2 and EP4 receptors. In addition, we demonstrate that SP augments the levels of nuclear ReLa in B. burgdorferi-stimulated microglia, suggesting a possible mechanism whereby SP augments B. burgdorferi-induced microglial responses.

Injury to the CNS in the form of trauma and/or infection can result in the initiation of robust inflammatory responses. Such responses may either be protective or result in progressive damage to brain tissue (12–14). Lyme neuroborreliosis occurs as a result of neurologic involvement during chronic Lyme disease, and has been shown to be associated with increased levels of proinflammatory molecules such as IL-6, and TNF-α within the CNS (22, 23). In this study, we show that SP can exacerbate B. burgdorferi-induced production of the inflammatory mediator, PGE2, by microglia in vitro. Furthermore, the ability of SP to enhance the expression of EP2 and EP4 receptors on microglia raises the possibility that augmented PGE2 secretion may act in an autocrine manner to further potentiate microglial function. As such, it is tempting to attribute these effects to the ability of SP to elicit activation of NF-κB, a transcription factor that plays a role in the induction of inflammatory molecules, such as COX-2 and PGE2. In such a scenario, SP may function to markedly augment inflammatory responses during CNS infections.

Although the role of PGs within the CNS is not well defined, studies have demonstrated that prostanoids are required to maintain homeostasis and plasticity in developing nervous tissues (46). However, a compelling body of evidence demonstrates that during inflammation, the levels of prostanoid production can change dramatically, indicating a role for these lipid mediators in the progression of inflammatory responses (46). It is, perhaps, not surprising that microglia cells constitutively produce PGE2 possibly through the actions of COX-1. However, levels of microglial-derived PGE2 were sensitive to both Bb and SP treatment and correlated with the induction of COX-2 by these cells. These results are consistent with other studies that demonstrate low constitutive prostanoid production that can be increased within minutes by inflammatory stimuli acting on constitutively expressed prostanoid synthetic enzymes (47). PGE2 can exert a variety of effects on many tissues through the interaction of this prostanoid with one of its four receptors. Although EP2 and EP4 receptors have been shown to modulate the function of cells such as macrophages (40, 48), and their expression can be induced by bacterial cell wall components such as LPS (49), the ultimate outcome of PGE2/EP receptor interactions is largely unknown. The presence of such receptors on microglia and the ability of Bb and SP to augment their expression may indicate a role for PGE2/EP receptor interactions during Bb-induced inflammation.

In summary, the present study identifies the neuropeptide, SP, as being a potent modulator of microglial responses in vitro and may contribute to an understanding of how this spirochete can initiate the damaging inflammation associated with Lyme neuroborreliosis.


