Development of Lyme Arthritis in Mice Deficient in Inducible Nitric Oxide Synthase

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Nitric oxide (NO) is a powerful antimicrobial agent and an important regulatory molecule of the innate immune response. To determine if NO has a role in experimental Lyme disease, arthritis-resistant DBA/2J and arthritis-susceptible C3H/HeJ mice were bred to be genetically deficient for inducible NO synthase (iNOS). Following footpad injection of Borrelia burgdorferi, arthritis was similar between iNOS-deficient and control animals regardless of their genetic background. Histologic examination and arthritis severity scores of ankles revealed no differences in arthritis development between iNOS-deficient and control animals. Despite being deficient in a key antimicrobial agent, iNOS-deficient mice had tissue levels of B. burgdorferi similar to those in control mice. Thus, NO does not have a critical role in susceptibility to Lyme arthritis through tissue damage via an overexuberant inflammatory response, nor is it required in resistance through the clearance of spirochetes from tissues.

Experimental inoculation of mice with the spirochete Borrelia burgdorferi results in an infection that partially mimics human Lyme borreliosis [1]. Within days, the spirochetes begin to disseminate from the site of inoculation and can subsequently be isolated from many tissues [1]. Susceptible mouse strains begin to develop joint swelling within the first few weeks of infection [1]. Antibody-mediated spirochete clearance leads to arthritis resolution a few weeks after infection [2]. Mice with chronic infections have sporadic reoccurrence of acute arthritis [1].

Genetic control of resistance and susceptibility to arthritis development appears to be complex. BALB/c mice are resistant when infected with low doses of bacteria; however, susceptibility to arthritis development increases with the increasing infectious dose [3]. Other resistant strains of mice, such as DBA/2 (DBA) and C57BL/6J, remain resistant even when inoculated with very high numbers of spirochetes [3, 4]. Susceptible C3H/HeJ mice, on the other hand, develop arthritis when infected with as few as 200 organisms [3]. Along with spirochete loads, many adaptive immune responses have been implicated in determining susceptibility to arthritis development. Antibody production by B cells is important for arthritis resolution [2]. Helper cell subsets appear to play a role in arthritis development, with Th1 responses correlating with susceptibility and Th2 responses correlating with resistance [5]. In hamsters, macrophages appear to play a direct role in arthritis development [6].

Nitrergic (NO) is an important inflammatory effector molecule produced by the enzymatic degradation of arginine by inducible NO synthase (iNOS) [7]. Expression of iNOS by macrophages results in a high output of NO, which has been shown in vitro and in vivo to be critical in cell-mediated defense against intracellular pathogens [8]. In addition to its bactericidal properties, iNOS was recently shown, along with interferon-α/β, to be a critical regulator of innate immunity to Leishmania major [9]. The outer surface lipoproteins (OspA and OspB) of B. burgdorferi and intact B. burgdorferi spirochetes induce NO production from macrophages and neural cells [10–12]. In vitro exposure to NO is detrimental to the immune system, suggesting a role for NO in controlling spirochete growth in vivo [10, 11].

We recently showed that NK cells are activated upon infection of arthritis-susceptible but not arthritis-resistant mouse strains [13]. NK cells are an integral part of the cytokine-inducible pathway, which leads to macrophage activation and NO production [14]. This suggests that susceptible mouse strains might have an overexuberant inflammatory response to borrelia infections, which may lead to excessive NO production and tissue damage resulting in arthritis. Another study examined the role of NO in experimental Lyme disease and found no involvement in arthritis. To circumvent this limitation, we have bred iNOS-deficient mice with both arthritis-susceptible C3H and arthritis-resistant DBA mouse strains to produce offspring that are unable to produce immunologically active NO.

Materials and Methods

Mice and infections. Female C3H/He, DBA/2, mice were purchased from Jackson Laboratory (Bar Harbor, ME). The wild-type
mouse strains were made iNOS-deficient by crossing them to C57BL/6J x 129 iNOS−/− mice (provided by John MacMicking and Carl Nathan, Cornell University, Ithaca, NY, and John Mudgett, Merck, Rahway, NJ) [16] and then back-crossing to the designated background. All mice were on at least the fifth generation back-cross and were between 4 and 6 weeks of age at the time of infection. B. burgdorferi N40 was provided by Steven Barthold (Yale University, New Haven, CT). For infections, an aliquot of frozen spirochetes was thawed, placed in 7 mL of BSK medium (Sigma, St. Louis) and grown for 5 days at 32°C. Mice were inoculated in both hind footpads with 5 × 10^7 B. burgdorferi in 50 μL of medium. All tibiotalars joints were measured weekly, using a metric caliper (Ralph’s Tool-A-Rama, South Plainfield, NJ) through the thickest anteroposterior diameter of the ankle. For tissue cultures, we aseptically collected blood, heart, spleen, urinary bladder, skin, and ankles and incubated them at 32°C for 14 days in BSK medium. Cultures were scored by placing 10 μL of supernatant on a microscope slide under a cover slip (22 × 22 mm) and examining 20 high-power fields by dark-field microscopy.

Histology. Mice were sacrificed 28 days following infection. Their ankles were washed with 70% ethanol, and the skin was removed. The sample was excised by cutting just above and below the ankle joint, and then it was placed in 10% buffered formalin. The sample was embedded in paraffin, sectioned, and stained with hematoxylin-eosin, using standard techniques. Arthritis severity scores were determined in a blinded manner and graded on a scale of 0–3 [17]. Grade 0 represents no inflammation, grades 1 and 2 represent mild to moderate inflammation, and grade 3 represents severe inflammation.

Competitive polymerase chain reaction (PCR). Ankles were excised as above and stored at −80°C. DNA extraction and competitive PCR were performed as described [4]. In brief, samples were digested in 0.5 mL of 1% collagenase for 4 h at 37°C. Following incubation, 0.25 mL of 3X lysis buffer (0.3 mg/mL proteinase K in 600 mM NaCl, 60 mM Tris-HCl [pH 8.0], 150 mM EDTA, 0.6% SDS) was added, and the mixture was incubated for 16 h at 55°C. The debris was pelleted, and the supernatants were transferred to new tubes. Sample DNA was extracted with phenol-chloroform and precipitated with ethanol. The sample DNA was then pelleted, washed with 70% ethanol, air dried, and resuspended in 0.2 mL of Tris-EDTA buffer. Competitive PCR was performed using a constant amount (0.25 pg) of the BC3 polycompetitor and ~150 ng of sample DNA. The BC3 polycompetitor consists of a linear DNA molecule containing modified portions of the fla and ospA genes of Borrelia species and a modified portion of the mammalian interleukin-4 (IL-4) gene [4]. The following sets of primers were used for PCR amplification of both wild-type and BC3 gene segments:

 ospA 5’ primer TCTTGAAAGGAGGTTAATCCTGCT, ospA 3’ primer CAAGTTTTGTTATTTTCAACTGCTGA; IL-4 promoter region (IL-4pr) 5’ primer GATCGACTGGACTAGGATGCGAG, IL-4pr 3’ primer GGGCCATACGCACCTCTCTCCTCCA.

Statistics. Results are expressed as means ± SD. Data were analyzed by use of analysis of variance followed by the Tukey test for multiple comparisons. For significance levels α = .05.

Results

We infected iNOS-deficient mice and their wild-type littermate controls in both hind footpads with 5 × 10^7 B. burgdorferi spirochetes. Footpad injections were used to deliver the spirochetes to a site near the joint of interest to avoid differences in arthritis development that might be due to various spirochete dissemination patterns in mice of different strains or immunologic deficiency. Injection of media alone into the footpad does not result in any histopathologic changes in the tibiotalar joints (Brown C, unpublished observations). Arthritis development was monitored for 28 days by weekly measurement of ankle diameters (figure 1). Both C3H and C3H iNOS-deficient mice developed severe swelling of both tibiotalar joints between 7 and 14 days of infection, and this swelling remained throughout the remainder of the experimental period. None of the DBA or DBA iNOS-deficient mice developed any redness or swelling of ankle joints during this time period. Ankles from C3H and C3H iNOS-deficient mice were often greater than that in their littermate controls, the differences were not statistically significant and likely secondary to other effects of iNOS deficiency.

To ensure that ankle swelling correlated with the underlying development of arthritis, we examined hematoxylin-eosin-stained sections of ankle joints. Histologic sections of mouse ankles pooled from seven separate experiments were scored in
a blinded manner for arthritis development on a scale from 1 (no inflammation) to 3 (severe arthritis) as described [17]. Mean arthritis severity scores for C3H (2.1 ± 0.6) and C3H iNOS-deficient (2.3 ± 0.5) mice were not different from each other but were significantly higher than those for DBA (1.0 ± 0.8) and DBA iNOS-deficient (0.9 ± 0.6) mice (P < .01). Thus, NO appears to play no role in either resistance or susceptibility to development of experimental Lyme arthritis.

Because NO is a powerful antimicrobial agent, we also investigated its effects on spirochete clearance. To compare spirochete burdens between C3H control and iNOS-deficient mice, we performed competitive PCR on DNA isolated from ankle joints 28 days after infection. Each reaction was spiked with a constant amount (0.25 pg) of the BC3 competitor to enable the relative assessment of *Borrelia* DNA in ankle tissues [4]. The upper bands in each lane are the BC3 amplification products, and the lower bands are wild-type DNA PCR products. The amount of mammalian DNA used in each sample was equalized using primers for a single-copy gene segment from the IL-4pr. Once the sample DNA was equalized, the adjusted levels were used, and the DNA was subjected to PCR amplification, using primers for *ospA*. Results of the PCR analysis (figure 2) showed relatively higher levels of *ospA* DNA in ankles of C3H than C3H iNOS-deficient mice. Results were similar in a repeat experiment, indicating that NO production is not required to limit spirochete numbers.

Spirochete burdens were compared in tissue culture samples obtained at the time of sacrifice from control and iNOS-deficient mice. Blood, hearts, spleens, urinary bladders, skin (ear punches), and ankles were aseptically removed from control and iNOS-deficient mice, and the tissues were cultured for 14 days in BSK medium. The cultures were then examined for the presence of *Borrelia* spirochetes. There were few differences between control and iNOS-deficient mice in the number of positive cultures for any given tissue. For example, in cultures of hearts from C3H mice, 2 of 9 were positive for *B. burgdorferi*, while 0 of 9 were positive for C3H iNOS-deficient mice. Similarly, 2 of 9 cultures of heart tissue from DBA mice were positive for spirochetes, while 3 of 9 were positive from DBA iNOS-deficient mice. Culture results for blood and spleen were similar to those for heart samples (data not shown). Bladder, skin, and ankles had the highest numbers of positive cultures, which is typical at this stage of the infection [4]. For example, 8 of 9 skin cultures for C3H mice were positive, 9 of 9 were positive for C3H iNOS-deficient mice, 8 of 9 were positive for DBA mice, and 8 of 9 were positive for DBA iNOS-deficient mice. Culture results from urinary bladder and ankle samples were similar to those from skin samples (data not shown). Thus, NO is not a critical mediator of spirochete clearance and may even inhibit efficient clearance of spirochetes by other effector mechanisms.

Discussion

In the present study, we infected mice deficient in NO synthase to investigate the role of NO in the development of experimental Lyme arthritis and spirochete clearance. The production of NO during immune responses has both protective and potentially pathogenic consequences [7]. NO production has been shown to be required for protection against infectious agents, such as *Listeria monocytogenes* [16] and *L. major* [9]. On the other hand, NO production has also been implicated as a key regulator of pathology caused by autoimmune diseases, such as rheumatoid arthritis and osteoarthritis [18]. Thus, NO production in response to infection with *B. burgdorferi* could be protective, by limiting spirochete burden, or exacerbative, by stimulating an overexuberant inflammatory response and subsequent tissue damage.

We infected iNOS-deficient mice on arthritis-resistant DBA and arthritis-susceptible C3H backgrounds and monitored arthritis development for 28 days. The development of arthritis was similar in control and iNOS-deficient animals of the same genetic backgrounds. Thus, NO production does not play a role in Lyme arthritis development. Spirochete cultures of specific tissues from control and iNOS-deficient mice revealed no difference in the presence of *B. burgdorferi*. PCR analysis of ankle tissue revealed slightly higher levels of spirochetes in the ankles of C3H compared with C3H iNOS-deficient mice. Thus, production of NO is not required to limit spirochete growth or to induce the clearance of spirochetes.

Our results confirm and extend those reported earlier by Seiler et al. [15]. In that study, C3H and BALB/c mice were infected intradermally with *B. burgdorferi* and treated with L-NMMA to inhibit NO production, and arthritis development was followed. Seiler et al. [15] found no differences in arthritis development, levels of spirochete loads in tissues, or antibody production between control and L-NMMA-treated mice. In the present study, we used footpad infection of mice; such infection delivers the spirochetes to a location directly adjacent.
to the joint to be studied, thus eliminating the need for spirochete dissemination. We also used mice deficient in iNOS, which rendered them incapable of NO synthesis. NO has a very short half-life, and it is possible that only minute amounts were needed at tissue levels that could not be blocked by L-NMMA treatment. Our results, however, agree with those from Seiler et al. [15] and indicate that the production of NO has no critical role in either resistance or susceptibility to development of pathology in experimental Lyme disease.

Acknowledgments

We thank Jennifer Bird and Nancy Reilly for technical assistance and John MacMicking and Carl Nathan (Cornell University) and John Mudgett (Merck Research Laboratories) for supplying the iNOS-deficient mice.

References