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*J Immunol* 2001; 166:7404-7409; doi: 10.4049/jimmunol.166.12.7404

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Gestational Attenuation of Lyme Arthritis Is Mediated by Progesterone and IL-4

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Infection of different strains of laboratory mice with the agent of Lyme disease, Borrelia burgdorferi, results in arthritis, the severity of which has been correlated with the dominance of Th1 cytokines. In this study, we demonstrate that changes in B. burgdorferi-specific immunologic responses associated with pregnancy can alter the outcome of Lyme arthritis in mice. Whereas nonpregnant female C3H mice consistently developed severe Lyme arthritis, pregnant mice had a marked reduction in arthritis severity that was associated with a slight reduction in IFN-γ and markedly increased levels of IL-4 production by B. burgdorferi-specific T cells. Similar reductions in arthritis severity and patterns of cytokine production were observed in nonpregnant, progesterone-implanted mice. Ab neutralization of IL-4 in progesterone-implanted mice resulted in severe arthritis. Our results are consistent with the known shift toward Th2 cytokine expression at the maternal-fetal interface, and are the first to show a pregnancy-related therapeutic effect in an infectious model. The Journal of Immunology, 2001, 166: 7404–7409.

Lyme disease is an infectious multisystem inflammatory disorder that is caused by the tick-borne spirochete, Borrelia burgdorferi. This disease is initially characterized by the enlargement of skin lesion around the site of the tick bite (erythema migrans); focal, intermittent inflammation involving myocardium, neurologic tissue, and/or joints may develop weeks to months after initial infection (1). In a subset of untreated cases, chronic arthritis ensues, which is associated with the persistence of small amounts of B. burgdorferi nucleic acids in synovial tissues and joint fluid (2).

In the murine model of Lyme disease, Th2 responses may protect against the development of arthritis because treatment of arthritis-resistant mice with anti-IL-4 increases arthritis severity, and treatment of susceptible mice with rIL-4 or passive transfer of CD4+ Th2 clones apparently reduces arthritis activity (3–7). Mouse strain-dependent resistance to arthritis is accompanied by a decrease in B. burgdorferi-specific IFN-γ production and serum IgG2a and IgG3 levels, along with an increase in IgG1 levels. Conversely, in chronically infected animals, Th1 responses appear to promote pathogenic inflammatory responses that may exacerbate arthritic activity, because administration of anti-IL-12 or anti-IFN-γ Abs reduces arthritis severity in susceptible animals (5, 6, 8). Th1-type cytokine production has also been reported in synovial T cells from patients with Lyme arthritis (9–11).

During pregnancy, a series of profound immunologic changes occurs, including changes associated with altered tryptophan metabolism and progesterone-mediated alterations in the balance of cytokine elaboration (12, 13). One of the generally observed effects on immune responses during pregnancy has been a bias toward humoral responses, often at the expense of cell-mediated immunity and associated inflammatory sequelae (14–19). Pregnant mice mount higher Ab levels to heterologous Ags compared with nonpregnant mice and display concomitant reductions in delayed-type hypersensitivity responses against paternal MHC and other nonself-Ags (20, 21). On the basis of these and other observations, it has been proposed that Th2 cytokines (IL-4, 5, 6, 10, and 13) produced at murine fetal-maternal interface down-regulate Th1 responses responsible for acute allograft rejection, thus along with other mechanisms, promoting fetal survival (18, 22, 23). However, this Th2 bias during pregnancy may have a deleterious effect on the outcome of certain infectious processes. In mice and in some instances in humans, pregnancy may increase susceptibility to certain intracellular pathogens such as Leishmania major (mice only), Listeria monocytogenes, Mycobacterium leprae, Mycobacterium tuberculosis, and Toxoplasma gondii (mice only), in which protective immunity is associated with Th1 responses (15, 16, 24–26).

In this study, we show that during pregnancy in a murine model, the severity of pathogenic inflammatory responses associated with Lyme arthritis is significantly attenuated. The pregnancy-associated reduction in disease severity was associated with modestly reduced production of IFN-γ and significantly higher relative production levels of the Th2 cytokine IL-4 compared with nonpregnant infected controls. This gestational therapeutic effect was reproduced in nonpregnant mice by progesterone treatment.

Materials and Methods

Mice

Inbred 4- to 5-wk-old female C3H.HeJ mice and 9-wk-old male BALB/c × SJL were purchased from The Jackson Laboratory (Bar Harbor, ME) and housed in the Mayo Clinic animal facility.

Spirochetes

B. burgdorferi N40 strain, originally obtained from S. Barthold, was used in all the experiments. Frozen aliquots of low passage N40 were thawed and grown to log phase in 10 ml modified Barbour Stoenner Kelly II (BSK)
Experimental infection in mice

Depending on the experiment, female mice were individually mated with stud BALB/c × SJL males. Evidence of vaginal plugs was considered day 0 of pregnancy. Control females were not mated. Mice were infected by subcutaneous inoculation behind the left shoulder intradermally. A total of 10⁷ spirochetes per animal in 100 μl of BSK II was used. At 19, 30, or 45 days (depending on the experiment), the animals were sacrificed, and regional draining lymph nodes and spleen were harvested for cytokine analysis. At every point, the infection status was assessed by culturing ear tissue in 10 ml BSK II medium at 32°C for 3 wk. The presence of viable spirochetes was assessed by dark-field microscopy at the end of the culture period.

Bb-specific IgG isotype ELISA

Ninety-six-well polystyrene plates were coated with whole cell lysates (1 μg/well) and kept at −20°C until further use. Plates were thawed and then blocked by the addition of 1% BSA/PBS Tween 20 solution for 1 h at 37°C. The cells were then washed extensively (PBS/Tween 20). Duplicate samples of murine serum (90 μl/well, 1:150) were applied to the plates and incubated for 1 h at 37°C. Goat anti-mouse (IgG1 and IgG2a), each diluted 1:4000 and linked to HRP (Southern Biotechnology Associates, Birmingham, AL), were added to each well, then incubated for 1 h at 37°C and washed. Then 100 μl of ABTS peroxidase (Kirkegaard & Perry Laboratories, Gaithersburg, MD) substrate was added and monitored at 405 nm. The reaction was stopped with SDS buffer.

Cytokine analysis

Regional lymph nodes and spleens were processed for IL-4, IL-10, IL-13, and IFN-γ analysis from pregnant/progesterone mice and from control, nonpregnant mice. Lymph nodes and spleens were individually processed into single cell suspensions in RPMI medium supplemented with 10% FCS, 100 U penicillin/ml, and 100 μg streptomycin/ml. Lymph node cells and splenocytes were washed with RPMI, resuspended in RPMI with 10% FCS and supplemented at 1 × 10⁵ cells/ml, and aliquoted into 96-well (total volume 200 μl) tissue culture plates. Lymph node cells and splenocytes were stimulated with B. burgdorferi sonicate at a final concentration of 25 μg/ml or with an equivalent volume of PBS. Supernatants were harvested at 72 h, IL-4, IL-10, IL-13, and IFN-γ were measured by a sandwich ELISA, as specified by the manufacturer (PharMingen, San Diego, CA). Concentrations for the previously mentioned cytokines were based on standard curves obtained from serial dilutions of recombinant IL-4, IL-10, IL-13, and IFN-γ.

Histopathology

The joints of hind limbs (tibiotarsal joints) were fixed in neutral-buffered Formalin (pH 7.2), dehydrated, and then processed and stained with hematoxylin-eosin by routine histologic techniques. The tibiotarsal joints were blindly scored for arthritis severity on a scale of 0 (no inflammation), 1 (mild, usually focal inflammation of synovium within the tibiotarsal complex, 2 (multiple sites of moderate inflammation without necrosis), and 3 (diffusely and severely inflamed lesions with areas of necrosis). The severity of synovial hyperplasia correlated with the intensity of inflammation. The histopathology of B. burgdorferi arthritis in the mouse has been thoroughly described (27, 28). Differences in mean values of arthritis severity in the different experiments were analyzed by Student’s two-tailed test.

Progestosterone implants

Mice were implanted s.c. with a 21-day time release progestosterone pellet (Innovative Research of America, Sarasota, FL). Briefly, while under methoxyflurane anesthesia (Medical Developments, Springvale, Australia), the hair on the back of the neck was clipped, a small (0.3-cm) incision was made, and a progestosterone pellet (25 mg/pellet) was inserted. Control animals received placebo pellets containing all the components of the progestosterone pellet, except the active product itself. The implants delivered 1.2 mg/day of progestosterone. This dose was based on the minimum amount of progestosterone required to maintain pregnancy (29). The incision was closed with silk, and the animals were maintained in individual cages.

Anti-IL-4 neutralization

Five milligrams of 11B.11 mAb (National Cancer Institute Biological Resources Branch, Frederick, MD) were administered i.p. on the day of infection, and were repeated weekly for 2 wk.

Quantitative B. burgdorferi detection by competitive PCR

B. burgdorferi DNA was purified from preweighed mouse ear tissue using a modified QIAamp tissue kit protocol (Qiagen, Valencia, CA) (30). A 256-bp region within the B. burgdorferi flagellin gene was used as a genomic target for amplification and quantitation. This mixture also contained 200 copies/μl of a 356-bp competitive internal control consisting of a 303-bp product from Staphylococcus aureus plasmid pUB112, tailed with the appropriate B. burgdorferi flagellin primer sequences. The PCR products generated during the amplification process were detected using a modification of the PCR ELISA detection kit (Roche Molecular Biochemicals; Indianapolis, IN) (30).

Results

Pregnant mice have mild Lyme arthritis

In three separate experiments, female, 6-wk-old C3H.HeJ mice were mated with BALB/c × SJL stud males and checked daily for evidence of vaginal plugs (day 0 of pregnancy). Within 24 h of plug development, 10⁴ spirochetes (strain N40) were inoculated intradermally at a consistent suprascapular location into the newly pregnant females (n = 37), and unmated controls (n = 25). Animals were followed up to day 19 of pregnancy and then euthanized. Tibiotarsal joints were collected and evaluated blindly for histopathologic determination of arthritis severity. Arthritis severity scores indicated a dramatic contrast between pregnant mice (mean score = 0.9; SD = 0.9) and control mice (mean score = 2; SD = 0.9) (Fig. 1). In most cases, pregnant infected mice showed mild infiltration of inflammatory cells in the tibiotarsal joint, whereas nonpregnant infected mice harbored moderate to severe infiltration (Fig. 2). Regional lymph nodes and spleens were collected and assayed individually for Th1 (IFN-γ) and Th2 (IL-4, IL-10, and IL-13)-specific cytokines. A moderate reduction of IFN-γ and a concomitant increase in IL-4 were observed in pregnant infected mice, whereas control nonpregnant mice demonstrated a predominant Th1-specific cytokine pattern (slightly higher IFN-γ production, little IL-4 production) (Fig. 3, A and B). We then examined serum levels of B. burgdorferi-specific IgG1 and IgG2a Abs to ascertain the relative influence of Th1 vs Th2 cytokines in vivo (31); a net increase in the ratio of the IgG2a subclass relative to IgG1 is associated with Th1-dominant immune responses. Consistent with the cytokine measurements, we observed a significant reduction of IgG2a levels in pregnant mice compared with nonpregnant controls (Fig. 4). No significant differences were observed for IgG1. All animals were proven to be infected by recovery of B. burgdorferi from a site distant from the

FIGURE 1. Effect of pregnancy on the arthritis severity in C3H mice. Histopathological scores on pregnant (✦) and nonpregnant (△) mice. Results represent individual animals from a representative experiment at day 19 of pregnancy (day 18 of infection).
site of inoculation. However, quantitative *B. burgdorferi* PCR performed on ear punch biopsy tissues indicated no significant differences in spirochetal tissue burden between pregnant and non-pregnant mice that might account for the differences in inflammatory activity (32) (data not shown).

**Timing of the anti-inflammatory effects of pregnancy**

To determine whether arthritis activity would rebound during the postpartum period, mice were mated and infected, as described initially in the previous experiments, but pups were removed immediately at time of delivery to allow progesterone hormone to approach pregestational levels (19–20 days after inoculation of *B. burgdorferi*). One cohort was followed up to day 30 after inoculation (10 days after removal of pups), and another cohort was followed up to day 45 postinoculation (25 days after removal of pups). Results for both cohorts indicated a sustained reduction in arthritis severity (30-day cohort, \( n = 15 \), mean arthritic score = 0.9; 45-day cohort, \( n = 15 \), mean arthritic score = 0.6). To determine whether the timing of infection relative to the onset of pregnancy determined the therapeutic effect of pregnancy, we infected, in two separate experiments, 6-wk-old C3H. HeJ females and mated them 7 days later. Animals that did not show vaginal plugs in the following 2 days were excluded from the experiment; thus, all animals were pregnant by day 8–9 after inoculation with *B. burgdorferi*. Mice (\( n = 16 \)) were followed up to day 19 of pregnancy (day 30 after inoculation); this time point was selected

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**FIGURE 2.** A, Normal tibiotarsal joint. Junction of tibia and tarsus. B, Detail of arrowed area in A. Uninflamed joint space, synovial lining, and adjacent soft tissues. C, Mild (1+1) acute inflammation limited to synovium. D, Detail of arrowed area in C. Edema and polymorphonuclear infiltrate in synovial tissues. E, Severe (3+) acute inflammation. Heavy polymorphonuclear infiltrates in synovium with mucoid inflammatory exudate in joint space. F, Detail of arrowed area in E.

**FIGURE 3.** Ag-specific cytokine production by regional lymphoid organs and spleen in *B. burgdorferi*-infected pregnant (\( n = 37 \)) and unmated control (\( n = 25 \)) C3H mice at day 19 of pregnancy (day 18 of infection). a, IFN-\( \gamma \); b, IL-4.
to coincide with the usual peak of arthritic activity. Again, pregnant infected mice showed a significant reduction in arthritis severity (mean arthritic score = 5.1; SD = 0.6) compared with non-pregnant controls (n = 12, mean arthritic score = 2.1; SD = 0.5).

**Progesterone mediates resistance to Lyme arthritis in pregnant mice**

To determine whether the protective effects of pregnancy were due to high levels of progesterone attained during pregnancy, we implanted 21-day progesterone time-released pellets s.c. into non-pregnant female mice (release rate of 1.2 mg/day). Mice were inoculated with 10^4 spirochetes 24 h after receiving the implant and followed up to day 19. Tibiotarsal joints, lymph nodes, and spleen were collected and processed as described. Arthritic scores indicated a significantly lower level of arthritis for mice with progesterone implants (n = 13, mean arthritic score = 0.7; control mice n = 10, mean arthritic score = 2.1) (Fig. 5). Cytokine profiles and IgG subclass determinations were equivalent to those obtained in pregnant mice (Fig. 6, A and B, and Fig. 7). Finally, to establish whether the observed differences in cytokine secretion in pregnant mice influenced the severity of arthritis, we treated progesterone-implanted mice with anti-IL-4 mAbs once per week for 3 wk (5 mg/wk). There was a significant increase in the severity of arthritis in the anti-IL-4-treated mice, closely matching the scores in non-pregnant, infected mice (Fig. 8).

**Discussion**

Pregnancy, in general, is associated with a general decline in production of Th1-associated cytokines and an increase in Th2-associated cytokines (33). Administration of Th1 cell-associated cytokines (IFN-γ or IL-2) or TNF-α is associated with immunologic rejection of fetal tissues, and can result in abortion (34, 35). Pregnancy also increases susceptibility to many diseases caused by helminths, protozoa, and bacteria, although this heightened susceptibility is most evident for infections with intracellular protozoa and bacteria (24–26). NK cells, Th1 T lymphocyte subsets, cytotoxic CD8^+ T cell activities, and the production of IFN-γ, IL-2, and TNF-α are all decreased during pregnancy and all contribute to the protective immune response against these pathogens (36). IL-4, IL-5, and IL-10, which down-regulate and antagonize the effects of IFN-γ, IL-2, and TNF-α, are produced locally at the maternal-fetal interface during pregnancy. This may explain the decreased production of IFN-γ observed in pregnant mice infected with *L. major* and *T. gondii* compared with nonpregnant mice (16, 37). Administration of IL-2 or IFN-γ to pregnant mice can significantly increase their resistance to *T. gondii* infection, but also increases the risk of abortion (38).

Ours is the first conclusive study showing decreased tissue pathology associated with an infectious process during pregnancy,
and is consistent with what is known about the pathogenesis of Lyme arthritis in inbred mouse models. We found little evidence of a stochastic effect leading to Th2-dominant responses during pregnancy, but rather an attenuation of the Th1 response and a corresponding decrease in inflammatory cytokine production.

Ag-stimulated lymphocytes from pregnant mice secreted higher IL-4 and lower levels of IFN-γ than nonpregnant controls. This differential cytokine secretion was noted in vivo, with the marked decrease in the IgG2a subclass of differential cytokine secretion was noted in vivo, with the marked decrease in the IgG2a subclass of the IgG2a subclass of B. burgdorferi Abs in pregnant mice compared with nonpregnant mice. We also examined regional (popliteal) lymph nodes in footpad-inoculated pregnant mice. The arthritis results as well as IFN-γ and IL-4 values were similar to animals infected by the suprascapular route (data not shown).

Interestingly, clinical observations in humans suggest that the severity of rheumatoid arthritis is ameliorated during pregnancy, whereas systemic lupus erythematosus, in which the principal pathology is associated with autoantibody production, may become exacerbated during gestation (39, 40). The delicate cytokine balance between the host response to infection and maintenance of pregnancy may also work against the fetus in some cases; host responses to certain pathogens, even if the site of infection is distant from the fetal-placental interface, may overwhelm fetal tolerance mechanisms and impair successful pregnancy (12).

Experimental infection of inbred mice with B. burgdorferi results in arthritis, the severity of which appears to be genetically determined by pathogen-host interactions that control the spirochete burden and/or the host inflammatory response (27, 28). B. burgdorferi-infected C3H mice characteristically mount a Th1-dominant response in the postacute phase, as evidenced by high levels of IFN-γ and low or undetectable levels of IL-4 in lymphocytes restimulated by B. burgdorferi Ags (3, 6). Consistent with this, B. burgdorferi-specific IgG2a serum levels exhibited a marked increase (6, 41). The arthritis-modulating effects of IL-4 have been shown by depletion of IL-4 with mAb and by experiments in which this cytokine has been administered exogenously during the course of B. burgdorferi infection of mice (3, 5, 6). The precise mechanism by which this cytokine is associated with joint inflammation has not been elucidated, but the known inhibitory effects of IL-4 on Th1-specific inflammatory cytokines could lead to a decrease in joint inflammation independent of direct effects on pathogen burden (42). Indeed, we did not detect significant differences in spirochetal tissue burden between pregnant and nonpregnant controls. In one study in which T cell cytokine secretion was monitored at different time intervals after initial inoculation during the development of murine Lyme arthritis, the evolution of a Th2-type immune response developed after an initial Th1-dominant response in a disease-resistant mouse (43), suggesting modulation of proinflammatory effects over time.

We used progesterone treatment of C3H mice to test the hypothesis that this hormone was responsible for the therapeutic effect of pregnancy (44, 45). In comparing pregnant mice with nonpregnant, progesterone-treated mice, our results showed similar degrees of reduced arthritis severity, differential cytokine secretion, and predominance of the IgG2a Ab subclass. We then observed a significant increase in arthritis activity when progesterone-treated animals were treated with anti-IL-4 Abs, indicating that the anti-inflammatory effects of progesterone were mediated by IL-4 in Borrelia-infected mice.

In conclusion, our study demonstrates that pregnancy alters the equilibrium of cytokine elaboration toward reduction of a pathogenic inflammatory response to an infectious challenge in pregnant mice. This down-regulation of Th1 responses, most likely via progesterone-mediated up-regulation of Th2 cytokine production, provides a plausible explanation for the significant reduction of Lyme arthritis in pregnant mice. Further clarification in the dynamics of the immune response in pregnant mice may in this, and in other infectious models, be useful for understanding the basis of the profound immunologic changes associated with pregnancy.

Acknowledgments

We thank T. Mossman, Yasir Skeiky, and Antonio Campos-Neto for helpful suggestions and discussions, and J. Hanson, L. Cummins, T. Trejo, and O. Zegarra-Moro for technical assistance. We also thank G. Reynolds from the National Cancer Institute for providing the 11B.11 mAb.

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