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Research Article

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Lyme Borreliosis in Transgenic Mice Tolerant to Borrelia burgdorferi OspA or B

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Abstract

The evolution of Lyme borreliosis in transgenic mice tolerant to Borrelia burgdorferi outer surface proteins (Osps) A or B was assessed to investigate the role of immunity to OspA or B in infection and pathogenesis of Lyme disease. Antibodies to OspA or B protect immunocompetent C3H/ HeJ or C.B.17 severe combined immunodeficient (scid) mice from challenge with B. burgdorferi. Moreover, arthritis in infected C3H mice resolves with the rise of high titers of B. burgdorferi specific antibodies, including OspA and B, whereas disease persists in scid mice—suggesting that the regression of arthritis may be due to the development of borreliacidal OspA or B antibodies. To evaluate the course of Lyme borreliosis in OspA or B tolerant mice we developed transgenic mice that expressed OspA or B under control of the major histocompatibility complex (MHC) class I promoter. Mice carrying OspA or B transgenes on a C3H/ HeJ (C3H, disease-susceptible) or C57BL/6 (B6, diseaseresistant) background, immunized with OspA or B, did not mount a humoral or cellular immune response to OspA or B, respectively, but responded normally to other B. burgdorferi antigens. The evolution of Lyme borreliosis, including infection and the development of arthritis and carditis, was similar in transgenic and nontransgenic littermates suggesting that an OspA or B immune response is not singularly involved in either the genesis or regression of Lyme disease in C3H or B6 mice. (J. Clin. Invest. 1995. 95:1706-1714.) Key words: Lyme disease • spirochete • arthritis • tolerance antibody

Introduction

Borrelia burgdorferi, a spirochete, causes Lyme disease—the most common vector-borne infection in the United States (1–3). Infection in man can result in a skin lesion, *erythema migrans*, and sometimes progress to cause disease involving the heart, joints and nervous system (2, 3). In most cases, the symptoms resolve but occasional exacerbations of disease may occur (2). Lyme borreliosis in inbred C3H/HeJ (C3H) mice partially mimics the human illness. C3H mice challenged with

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B. burgdorferi N40 develop a disseminated infection with spirochetemia, arthritis and carditis that is most severe at 2-4 wk (4, 5). At these time points the inflammatory response is marked by a synovial exudate with fibrinoid necrosis and a neutrophilic infiltrate (5). Over a period of several months the disease progresses to a lymphoplasmacytic infiltrate and then diminishes, usually by 6 mo, after which time only residual scar tissue is evident in the joints and heart (5, 6). Rarely, exacerbations of acute inflammatory arthritis, in association with recurrent spirochetemia, can occur in the chronically infected mouse (5). Although the disease manifestations of Lyme borreliosis in the C3H mouse are mostly transient, the animals remain persistently infected and B. burgdorferi can be cultured from selected murine tissue from 5 d to over 1 year after the initial infection (5, 6). The course of Lyme borreliosis in humans and mice has been clearly described; however, the factors contributing to the pathogenesis of disease are likely to be multifactorial and remain to be determined.

The pathogenesis of Lyme arthritis is partially influenced by the host's genetic background and ability to clear the spirochete infection. Steere and his colleagues showed that the presence of the HLA-DR4 allele and high antibody titers or cellular immune responses to OspA or B in patients correlated with an increased susceptibility of developing chronic arthritis that is resistant to antibiotic therapy (7-9). These studies have led to the proposal that the immune response to OspA may be directly involved in the pathogenesis of disease. Moreover, Peltz and his associates identified T_h1 T cell clones that responded to OspA in Lyme arthritis synovial fluid and postulated that the cellular immune response to OspA contributes to the genesis of disease (10, 11). Peltz's group then further demonstrated that cellular reactivity to diverse epitopes of OspA, and expansion of the V β 5 T cell population, is evident in patients with Lyme arthritis (12, 13), While these studies suggest that OspA or B specific immune reactivity is associated with the development of arthritis, a cause and effect relationship between an OspA or B response and disease has not been established. These data lead to the hypothesis that the ablation of the host's ability to respond to OspA or B would lead to a reduction in the severity of arthritis.

Inbred strains of mice also develop varying degrees of arthritis following infection with *B. burgdorferi*—further suggesting that genetically determined host susceptibility contributes to disease. Schaible and his colleagues showed that mice of the H-2^k haplotype are more susceptible to clinically evident Lyme arthritis than mice with the H-2^d allele, and postulated that a cellular immune response to *B. burgdorferi* antigens causes disease (14). Barthold also showed that C3H mice (H-2^k) infected with *B. burgdorferi* developed severe acute arthritis—by histopathologic examination of the joints—that regressed over a period of 1 to 2 mo whereas B6 mice (H-2^b) developed less pronounced disease that followed a similar time course (4). Furthermore, Weis and her associates have shown that the severity of arthritis in inbred mice is partially accounted for by a

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greater number of spirochetes in the joints of the animals with marked disease (15). These studies all suggest that the major histocompatibility complex (MHC) haplotype contributes to the genesis of disease and that arthritis may be related to an antigenspecific immune response to *B. burgdorferi*, to the quantity of spirochetes in the infected host, or both. In contrast to these murine models of acute regressing arthritis, C.B.17 severe combined immunodeficient (*scid*) mice develop persistent unremitting arthritis suggesting, first, that a protective immune response is important in the regression phase of disease and, second, that a *B. burgdorferi* specific response is not necessary for the genesis of Lyme disease in *scid* mice (16, 17).

We, and others, showed that immunization of mice with OspA elicits a protective immune response (18, 19). Humoral immunity was sufficient for protection as passive immunization with OspA antibodies also prevented infection in both immunocompetent and immunodeficient mice (18, 20). Vaccination with OspB or C can also elicit partially protective immunity; however, the effects are less pronounced than with OspA (21-23). Moreover, adsorption of OspA or B antibodies from protective rabbit anti B. burgdorferi serum or sera from patients with Lyme disease substantially reduces the protective capacity of the serum, further indicating that OspA and B play major roles in the development of immunity (21, 24). Mapping studies showed that neutralizing OspA or B antibodies bind to regions within the carboxyl terminus of OspA or B, thereby implicating these regions of the antigens in protection (22, 25). Indeed, during natural infection, arthritis resolved as C3H mice mounted a humoral response to OspA that included antibodies that bind to protective epitopes (25). These data suggest that the diminution of murine arthritis may be related to the host's ability to generate an OspA or B specific immune response.

The studies in mice and humans show that the pathogenesis of Lyme disease is multifactorial. Indeed, in numerous infectious diseases, the immune response to a pathogen, rather that the pathogen itself, is involved in the development of disease. Several reports indicate that the murine or human MHC haplotype may contribute to Lyme disease-either because of an inability of the host to control the spirochete or due to inflammation mediated by an immune response directed towards specific B. burgdorferi antigens, such as OspA and B. In contrast, other studies suggest that OspA and B antibodies are important for killing spirochetes and that the development of a borreliacidal immune response during infection contributes to the regression of disease. As studies implicate the OspA or B immune response, both directly in the genesis of arthritis and in the establishment of immunity and disease modulation we now evaluate Lyme borreliosis in disease-susceptible (C3H) and disease-resistant (B6) mice in which the OspA or B response has been selectively ablated. To determine how the host's inability to selectively respond to OspA or B influences the course of murine Lyme borreliosis we generated OspA or B transgenic mice that are tolerant to OspA or B respectively, and assessed the evolution of infection and disease in the transgenic animals.

Methods

Generation of OspA or B transgenic mice. To develop the OspA or B transgenic mice we expressed OspA or B under control of the MHC class I promoter that constitutively expresses class I molecules on virtually all nucleated cells. To direct transcription of ospA or ospB we used the plasmid H-2K^bpBR327 (pH-2K). This plasmid is a pBR327-based construct that contains the mouse MHC class I gene of the H-2K^b allele,



Figure 1. The recombinant plasmids expressing OspA (A) or OspB (B) under control of the class I promoter. P contains the promoter region and E1-E8 represent exons 1 through 8 of the H-2K^b gene. EcoRI, HindIII and ApaI restriction enzyme digestion sites and the pBR327 sequence are shown. The *ospA* gene (nucleotides 52-819, without the *ospA* signal sequence) or the *ospB* gene (nucleotides 49-888, without the *ospB* leader sequence) were inserted into an ApaI site in exon 2, after the class I leader sequence. The plasmids have been linearized for clarity.

including the promoter, exons 1 through 8 and a polyadenylation sequence, within a 10-kb fragment flanked by EcoRI restriction enzyme digestion sites. Exon 1 encodes the 5' untranslated region and leader sequence. Exons 2, 3, and 4 contain the α 1, 2 and 3 domains of the class I molecule, respectively. Exon 5 encodes the transmembrane region, exons 6 and 7 encode the cytoplasmic domains, and exon 8 contains the 3' untranslated region. The class I promoter has been used for the expression of other antigens in transgenic mice (26) (Fig. 1).

ospA or B were cloned into pH-2K to create the OspA plasmid construct used for injection into mouse embryo (Fig. 1). ospA or B from B. burgdorferi N40 was amplified using the polymerase chain reaction (PCR). 10 ng of a pGEX-2T based plasmid containing ospA or B was used as the template (18, 22). Primers contained 20 nucleotides of ospA or B. For ospA the 5' primer started at nucleotide 52, immediately after the ospA leader sequence and the 3' primer began at position 819, which is the final nucleotide in ospA. The ospB 5' primer began at nucleotide 49 (after the ospB leader sequence) and the 3' primer started at the end of ospB, nucleotide 888. The PCR primers were flanked by ApaI restriction enzyme digestion sites to facilitate subcloning. PCR was performed for 30 cycles with a denaturing temperature of 94°C, an annealing temperature of 40°C and an extension temperature of 55°C. The amplified PCR products were purified by electroelution onto a DEAE membrane and extraction into a 1 M NaCl solution. The purified ospA or B DNA was digested with Apa 1 and ligated into the pH-2K plasmid construct that had been digested with ApaI. The ospA or B gene was inserted into the Apa 1 site at position 166 within exon 2 so that the gene would be expressed as a translational fusion protein with the class I leader sequence, beginning at the amino terminus of the protein, under control of the class I promoter. The mixture was electroporated into Escherichia coli strain DH5 α , and positive colonies identified on Luria Broth medium with ampicillin. Both strands of the ospA or B constructs were sequenced to insure that the ospA or B inserts were correctly positioned in the appropriate orientation and reading frame and that mutations had not been incorporated during PCR amplification. A TAA stop codon was inserted after ospA or B to insure that a hybrid Osp-class I antigen was not expressed. The ospA or B leader sequences (nucleotides 1-51 and 1-48, respectively) were eliminated to increase the likelihood of subsequently developing a secreted form of the protein: it is probable that the additional hydrophobic bacterial leader sequence, which has a lipid insertion site, would result in an OspA or B that is retained within the endoplasmic reticulum of cells in the transgenic mice because of improper folding.

We injected the *ospA* or *B* constructs into C3H/HeJ (C3H) or (C57BL/6 [B6] \times CBA)F2 day 1 embryos—C3H embryos were used because C3H mice are highly susceptible to *B. burgdorferi* induced

arthritis, and (B6 \times CBA)F2 mice can be used to generate animals that are predominantly B6 (relatively resistant to B. burgdorferi induced arthritis) by selective breeding. By using these two strains of inbred mice we could then determine the effects of OspA or B tolerance on disease pathogenesis in mice that develop different degrees of arthritis. To prepare the DNA for microinjection into mouse embryos, the recombinant plasmids were purified by double-banding the plasmids over a CsCl gradient by equilibrium centrifugation. The EcoRI fragment of plasmid DNA was linearized by partial digestion with EcoRI. The 10kb fragment was isolated on an agarose gel, purified, concentrated using an Elutip-d column and dialyzed into a buffer suitable for microinjection of DNA into murine blastocysts (0.01 M Tris pH 7.4, 0.0001M EDTA, and pyrogen free water). The micro-injected embryos were implanted into pseudo-pregnant females and newborn mice were analyzed for the presence of ospA or B by Southern blotting. DNA was isolated by digestion of mouse tail specimens with proteinase K overnight at room temperature, and then purified by phenol chloroform extraction. 10 μ g of the recovered DNA was used for further analysis. Slot blots were performed on the isolated DNA using a ^{32}P labeled ospA or B probe. The ospA or B probes contained the entire ospA or B sequences and were labeled using the random Prime-It kit (Stratagene, La Jolla, CA). Specific transgenic lines in B6 mice were expanded by the selective breeding of the $(B6 \times CBA)F2$ founder mice with B6 mice for six generations. When necessary, as was the case with OspB, (B6 \times CBA)F2 founder mice were backcrossed with C3H partners for six generations to create OspB transgenic mice that were predominantly of the C3H strain.

B. burgdorferi. Cloned *B. burgdorferi* N40 with proven pathogenicity in mice were cultured in Barbour- Stoenner-Kelly II (BSK II) media (24). The concentration of organisms was adjusted to 1×10^5 or 1×10^8 spirochetes per ml. Mice were inoculated with 1×10^4 or 1×10^7 organisms in 100 μ l of BSK II media intradermally.

ELISA. B. burgdorferi N40 whole cell lysates or recombinant OspA or B was used as the substrate for ELISA (27). Recombinant OspA, B or the whole cell lysate were diluted to a concentration of 1 μ g/ml in 0.05 M sodium carbonate pH 9.6. Triplicate sets of 96-well microtiter plates were coated with the prepared antigen and then incubated overnight at 4°C. Plates were then washed three times with 0.05% phosphate buffered saline with 1% Tween (PBST). 200 μ l per well samples of murine sera (diluted 1:1,000) in PBST were applied to coated plates, incubated for 45 minutes at 37°C, and washed three times with PBST. Goat anti-mouse IgM or IgG conjugates (Sigma Chemical Co., St. Louis, MO) diluted 1:1,000 in PBST (200 μ l per well) were then applied and incubated for 45 min at room temperature. After washing three times with PBST, 200 μ l of freshly prepared p-nitrophenol in glycine buffer pH 10.5 was added to each well and incubated for 1 h and the reaction was stopped with 50 μ l of 3 M NaOH. The optical densities of the test sera were then plotted using pooled sera from 4 to 6 normal mice as the negative control.

Immunoblots. 1.5 μ g of B. burgdorferi whole cell lysates or recombinant OspA or B were resolved in a 12% polyacrylamide gel (Hoeffer, San Francisco, CA) (22, 24, 27). Proteins were transferred to nitrocellulose paper and the resulting strips were blocked in 5% bovine serum albumin (BSA) for 90 min and then incubated with murine serum (1:1,000 dilution) in 5% BSA for 1 h. The strips were washed three times with PBS and bound antibody was detected by incubation with goat anti-mouse IgG or IgM secondary antibody-conjugated to horseradish peroxidase using the enhanced chemiluminescence immunoblotting detection system (Amersham, Arlington Heights, IL).

Immunization studies. To determine functional immune tolerance to OspA we measured OspA antibodies in OspA transgenic mice immunized with recombinant OspA in complete Freund's adjuvant (CFA). Identical studies were performed using OspB transgenic mice and OspB as the immunizing antigen. Since ospA or B in the transgenic mice did not contain the osp leader sequences, the amino acids that correspond to these regions were not included in the recombinant OspA or B used for immunization. The OspA from B. burgdorferi N40 used for immunization was prepared as a nonlipidated antigen and purified in a fashion identical to the work of J. Dunn (28). The OspB immunogen was

prepared as a glutathione transferase fusion protein in which the OspB leader sequence (aa 1–16) was not included. OspB was cleaved from the glutathione transferase fusion partner using thrombin, which acts at a thrombin cleavage site engineered into the construct (22). Mice were subcutaneoulsy immunized with 10 μ g of recombinant OspA or B in CFA (100 μ l) and boosted twice at 14-d intervals with an identical amount of antigen in incomplete Freund's adjuvant (IFA) (21). Non-transgenic littermates were immunized in the same way and served as controls. The OspA or B transgenic mice were immunized with OspA or B antibodies on days 5, 14, 28, 35, 42, 72, or 105.

Infection studies. Groups of 5 OspA or B transgenic mice and nontransgenic littermates were challenged with an intradermal inoculation of 10⁴ or 10⁷ B. burgdorferi N40 in 100 µl of BSK II medium, according to our published methods (21). Mice were killed from 14 to 180 d after infection. Joints (both knees and tibiotarsi) and hearts were formalin fixed, paraffin embedded, sectioned and blindly examined microscopically for evidence of inflammation, as previously described (29). Arthritis was blindly graded on a scale from 0 to 3. Grade 0 represented the lack of inflammation, grades 1 and 2 indicated mild to moderate inflammation and grade 3 signified severe inflammation. Active arthritis was scored, tabulated and distinguished as acute (active) or chronic (inactive). Active arthritis was marked by exudation of fibrin and neutrophils into the joints, tendons and ligament sheaths, and hypertrophy and hyperplasia of the synovium. The grade of arthritis was based upon the extent of the observed inflammatory changes. Resolving lesions, with focal lymphoplasmacytic infiltration and synovial scarring, but without active exudation were categorized as chronic, inactive, lesions. Carditis was active when there was acute neutrophilic arteritis of the aortic adventitia and chronic when there was lymphoplasmacytic infiltration of the aorta and connective tissue of the heart base. Blood, spleen, and bladder were collected from the mice, cultured in BSK II medium, incubated for 2 wk, and examined by darkfield microscopy for spirochetes. Twenty high power fields were scanned per culture and the presence of 1 or more B. burgdorferi was considered positive.

Northern blot. Northern blot analysis was performed on mRNA isolated from selected murine tissue from the transgenic mice using the FastTrack mRNA isolation kit (Invitrogen, San Diego, CA). Spleen, liver, thymus, kidney, brain, and lung (1 gram) were removed immediately upon sacrifice and cut into small pieces (0.5 cm). 15 ml of lysis buffer (5 M guanidinium isothiocyanate, 0.05 M Tris-HCl pH 7.5, 0.01 M Na₂EDTA, 5% β -mercaptoethanol) was added to each tissue specimen and homogenized. The lysate was filtered through a sterile plastic syringe fitted with an 18-gauge needle and incubated at 40°C for 30 min. An oligo dT cellulose tablet was added to the lysate for 15 min at room temperature, pelleted, washed three times and resuspended in low salt wash buffer. RNA was then precipitated and stored at -70° C until use. For northern blot analysis, RNA was subjected to electrophoresis on a formamide gel, transferred to nitrocellulose, and probed using ³²P labeled *ospA*.

Proliferation assays. To assess cellular immune responses to OspA or B in the transgenic mice, animals were immunized with 10 μ g of B. burgdorferi lysates in CFA. Lymph nodes were harvested from mice 9 d after immunization. Suspensions of lymph node cells were prepared in Bruff's medium with 5% fetal calf serum, and adjusted to a concentration of 5 × 10⁶ cell/ml. 100 μ l of the cells (5 × 10⁵ cells) were placed in triplicate wells in 96-well flat bottom plates. 100 μ l of Bruff's medium containing antigen (OspA, OspB, or B. burgdorferi whole cell lysate, all at 10 μ g/ml—a dose that elicits optimal cell proliferation) was added to each well and incubated at 37°C for 4 d. On day 4, 1 μ Ci of [³H]thymidine was added to each well and incubated for 24 h. The wells were then harvested and the incorporation of radioactivity measured using a β counter.

Results

Development of OspA or B transgenic mice. Several lines of transgenic mice were generated that expressed ospA or B under



Figure 2. ospA mRNA expression in OspA transgenic mice. mRNA from selected organs was probed with ospA. Lane 1, liver; lane 2, spleen;

lane 3, thymus; lane 4, brain; lane 5, lung; 6, heart; lane 7, spleen from a nontransgenic littermate (control).

the control of the MHC class I promoter. Transgenic mice were generated on an inbred C3H or $(B6 \times CBA)F2$ background and subsequently backcrossed using B6 or C3H partners so that the influence of tolerance to OspA or B could be assessed in mice that develop severe (C3H) or mild (B6) arthritis. Two founder lines of OspA or B transgenic mice, respectively, were created using $(B6 \times CBA)F2$ embryos. The four founders were backcrossed for six generations using B6 partners to obtain mice that were on a predominantly B6 background. OspB founder mouse No. 1 was also backcrossed for six generations with C3H partners to generate an OspB transgenic mouse that was predominantly C3H. As expected, all mice backcrossed using B6 partners expressed the H-2^b haplotype, and mice backcrossed using C3H partners expressed the H-2^k allele. One OspA transgenic founder was created directly from C3H embryos. These mice were then used in infection studies to assess the effect of the transgene on the evolution of Lyme borreliosis in mice that develop severe (C3H) or mild (B6) disease. All the mice were viable, healthy, free of disease, and fertile.

To determine if the mice expressed the incorporated transgene, Northern blot analyses were performed on mRNA isolated from selected tissue from 3-wk-old OspA transgenic mice. RNA from the spleen, liver, thymus, lung, heart and brain were probed with ³²P labeled *ospA* DNA. mRNA expressing the *ospA* transgene was detected in the spleen, thymus, liver, and kidney, as a band slightly > 2 kb. By far, the largest amount of *ospA* was evident in the spleen (Fig. 2). The class I promoter is inactive, or weakly active in the brain, and as expected, transcription of *ospA* was not detected in this tissue. *ospB* mRNA was expressed in OspB transgenic mice in a similar fashion (data not shown).

Immune tolerance in OspA- or B-transgenic mice that were immunized with OspA or B. To determine if the OspAtransgenic mice were unable to make OspA antibodies, we hyperimmunized the mice with recombinant OspA. Transgenic mice on both the C3H or B6 background were given 10 μ g of recombinant OspA in CFA, and boosted twice at 14-d intervals with an identical amount of antigen in IFA. Nontransgenic littermates were treated in the same way and served as control animals. All the OspA transgenic mice were assayed for OspA antibodies up to 105 d after immunization (Fig. 3 A). Nontransgenic littermates readily developed OspA antibodies by 21 d after the initial immunization and the levels plateaued by 42 to 72 d after immunization, as shown in Figs. 3 A and 4 C (In Fig. 4 C, recombinant OspA migrates at a lower molecular mass than native OspA because of removal of the OspA leader sequence during preparation, resulting in a lack of lipidation). In contrast, the OspA transgenic mice did not develop significant detectable levels of OspA antibodies (Fig. 3 A). It is likely that the low levels of antibody identified by ELISA (Fig. 3A) at the later time points in some of the OspA transgenic mice immunized with OspA represented antibodies to E. coli products that were retained with the recombinant OspA during protein

purification because sera from immunized mice showed reactivity to a lysate of E. coli but not to purified recombinant OspA in immunoblot (Fig. 4A). The tolerance observed was specific for OspA as the OspA transgenic mice developed antibodies to OspB, both in the recombinant and native form, after immunization with recombinant OspB (Fig. 4 B). Reactivity with recombinant OspB was more obvious because of an increased amount of OspB antigen present on the immunoblot, rather than the level of native antigen in B. burgdorferi lysates. Recombinant OspB has a lower molecular mass than native OspB because of the removal of the OspB leader sequence resulting in a lack of lipidation. In addition, the antibody titers to whole cell lysates of B. burgdorferi by ELISA was similar in OspA transgenic mice and nontransgenic littermates (controls) immunized with 10 μ g of heat-killed spirochetes in CFA—in both cases, antibodies to B. burgdorferi were detectable at a serum dilution of 1:100,000.

Similar studies were performed with the OspB-transgenic mice generated on both a C3H or B6 background. The OspB transgenic mice were immunized with recombinant OspB, in an identical fashion as the OspA transgenic mice that had been immunized with OspA. OspB transgenic mice did not make antibodies to OspB, as detected by ELISA, whereas the control mice had high levels of OspB antibodies (Fig. 3 B). Moreover, analogous to the studies with OspA, some of the low levels of antibodies generated after immunizing the OspB transgenic mice with OspB may have been directed toward residual E. coli products retained during the purification of OspB. Tolerance was specific for OspB, as the OspB transgenic mice developed antibodies to OspA, both in the native and recombinant form, following immunization with OspA (Fig. 4 D). In addition, antibodies to B. burgdorferi lysates, after immunization with heat-killed spirochetes, could be detected in both OspBtransgenic mice and nontransgenic littermates at a dilution of 1:50,000 by ELISA.

Immune tolerance in OspA or OspB transgenic mice that were infected with B. burgdorferi. OspA- or B-transgenic mice were then infected with B. burgdorferi to determine if the antigen-specific tolerance observed after immunization was also evident following infection. The humoral response to B. burgdorferi after challenge with 10⁴ spirochetes over a period of 2-4 wk was similar in nontransgenic and transgenic mice and detectable by ELISA using a whole cell lysate of *B. burgdorferi* as the substrate at a dilution of 1:1,000. As expected with a low challenge dose of spirochetes, the control mice did not develop an antibody response to OspA or B at these early time points following infection. At later time points (2 to 6 mo) antibody titers to whole cell lysates of B. burgdorferi were detectable by ELISA at a dilution of 1:10,000 in both the transgenic and control mice. Although an antibody response to OspA was detected in the nontransgenic littermates, OspA transgenic mice did not develop an OspA response (Fig. 5 A and B). Likewise, in similar studies, OspB transgenic mice did not generate an OspB response (Fig. 5 C). When the OspA or B transgenic mice were challenged with a larger dose of spirochetes (10^7) , the control animals developed a more rapid response to OspA or B-sometimes detectable as early as 1 month after infection. Nevertheless, complete antigen-specific ablation of the OspA or B antibody response was still evident in the transgenic mice.

The cellular immune responses to OspA and B in the OspA or B transgenic mice were evaluated in proliferative assays (Table I). OspA or OspB transgenic mice, and nontransgenic littermates were hyperimmunized with whole cell lysates of



Days after immunization

Days after immunization

Figure 3. (A) OspA antibodies (IgG) in OspA transgenic mice (B6 or C3H) that were immunized with OspA. B6-1, B6-2, and C3H-1 represent mice from separate founder lines. Controls are the accumulated data from 4 nontransgenic littermates (C3H or B6) immunized with OspA. (B) OspB antibodies (IgG) in OspB transgenic mice (B6 or C3H) that were immunized with OspB. B6-1, B6-2, and C3H-1 represent mice from separate founder lines. Controls are the accumulated data from 6 nontransgenic B6 littermates immunized with OspB.

B. burgdorferi in CFA, and the harvested lymph nodes were stimulated with OspA, B, or B. burgdorferi lysates. OspA- or B-transgenic mice, and nontransgenic littermates had similar responses to whole cell lysates of B. burgdorferi, indicating that all the animals were capable of eliciting equivalent degrees of cellular reactivity, with stimulation indices of 8.4, 9.8, and 7.3, respectively. OspB-transgenic mice were not able to mount a proliferative response to OspB whereas OspA transgenic and control mice developed similar proliferative responses to OspB with stimulation indices of 2.8 and 2.5, respectively. In addition, OspA-transgenic elicited a blunted proliferative response to OspA (stimulation index 2.1), when compared with the nearidentical strong response to OspA that were evident in the OspB transgenic and control mice (stimulation indices of 13.5 and 10.8, respectively). Moreover, proliferative responses to OspA were not noted in assays which were performed with purified T cells, isolated from OspA transgenic mice that were immunized with B. burgdorferi lysates (stimulation index 0.3, cpm 542). Purified T cells from nontransgenic mice, immunized with B. burgdorferi lysates, proliferated in response to OspA (stimulation index 8.4, cpm 3545).

Lyme borreliosis in OspA or B transgenic mice. To determine the effect of tolerance to OspA or B on the evolution of infection and disease in the transgenic animals, mice were inoculated with B. burgdorferi. OspA- or B-transgenic and nontransgenic littermates, generated on both a background that is highly susceptible to arthritis (C3H) and on a background that develops less severe joint disease (B6), were infected with moderate (10^4) or large doses (10^7) of *B. burgdorferi* N40 delivered via an intradermal inoculation. The animals were sacrificed between 14 and 180 d after challenge, time points that represent different stages in the evolution of murine Lyme borreliosis (5, 6).

OspA transgenic mice, backcrossed onto a B6 background developed mild arthritis that was similar to the disease in nontransgenic littermates (Table II). Acute arthritis and carditis, with a marked synovial exudate and neutrophilic infiltrate was evident 14 to 30 d after challenge with 10⁴ spirochetes, and the degree of inflammation was mild (severity 0.8 [mean]±0.2 [standard deviation] to 1.1 ± 0.3 on a scale from 0 to 3). In general, disease was more readily apparent in the tibiotarsi than the knees. Arthritis began to evolve to a lymphoplasmacytic infiltrate and then resolve in the OspA transgenic mice and nontransgenic littermates at 60 d; the rate of disease regression was similar in both the OspA transgenic mice and controls. From 120 to 180 d after infection, residual scarring was evident. Exacerbations of disease were not noted in either group of mice. Carditis, though not graded in severity, was present in OspA transgenic and control mice at early stages after infection in a similar prevalence. Moreover, the rate of recovery of spiro-





Figure 4. Antibody responses (IgG) in OspA- or B-transgenic mice. All immunoblots are examples of sera from individual mice, and representative of blots performed on at least 10 separate mice. (A) Antibodies in sera from OspA transgenic mice immunized with OspA to lysates of *E. coli* (lane 1) and recombinant OspA (lane 2). (B) antibodies in sera of OspA transgenic mice, immunized with OspB to *B. burgdorferi* OspB (34 kD) (lane 1) or recombinant OspB (lane 2). (C) OspA antibodies in the sera of nontransgenic littermates (controls) immunized with OspA to recombinant OspA (lane 1) and native OspA (lane 2) in a whole cell lysate of *B. burgdorferi*. (D) Antibodies in sera of OspB transgenic mice, immunized with OspA to *B. burgdorferi* OspA (31 kD) (lane 1) or recombinant OspA (lane 2).

chetes from blood, spleen and bladders from OspA transgenic mice (89%) and control mice (90%) was similar throughout the course of infection.

To determine if the challenge inoculation of spirochetes could affect the course of disease, the mice were also challenged with a large dose of *B. burgdorferi* (10^7) . The evolution of disease was similar in both the control and experimental transgenic mice challenged with this number of organisms.

The evolution of Lyme borreliosis in OspA transgenic C3H mice, an inbred strain that is highly susceptible to the development of severe arthritis was also examined. As expected, C3H mice developed more pronounced arthritis that B6 mice, with moderate inflammatory arthritis (severity score of 1.7 ± 0.4 to 1.9 ± 0.3) 14 to 30 d after challenge. The degree of arthritis, the course of resolution of the joint and heart disease, and ability to recover spirochetes from OspA transgenic mice and non-transgenic littermates was similar. In addition, increasing the

Probed with sera from an OspB-transgenic mouse (Lane 1) or a non-transgenic littermate (lane 2).

with B. burgdorferi for 12 wk. (C).

challenge dose of spirochetes to 10^7 did not result in noticeable differences in the course of Lyme borreliosis between the control and experimental transgenic mice.

The course of Lyme borreliosis in OspB transgenic mice, on both the B6 and C3H background was also similar in the experimental and control mice (Table III). 67 OspB transgenic mice were individually assessed in these studies. In OspB transgenic mice on a B6 background, arthritis was mild 0.5 ± 0.3 at the 14- and 30-d time points and then resolved from 60 to 180 d. Somewhat fewer joints were affected with arthritis throughout the course of the study (14 to 180) days in OspB transgenic mice when compared with control animals; however, the differences were not statistically significant. In addition, carditis and the recovery of spirochetes from the transgenic and control mice was similar and increasing the challenge dose of spirochetes to 10^7 did not result in differences in the evolution of disease. As expected, arthritis was more severe in OspB transgenic mice on a C3H than a B6 background, and a greater number of the joints showed evidence of disease. Nevertheless, a similar course of infection and disease was noted within the control group as well.

Discussion

This study assesses the course of Lyme borreliosis in transgenic mice tolerant to OspA or B. Previous studies have implicated the immune responses to OspA and B in the pathogenesis of Lyme arthritis, both in animal models of disease and in patient populations (7, 10-12, 30-32). For example, OspA and B

Table I. Lymph Node Cell Proliferative Responses in OspA- or B-transgenic Mice Immunized With B. burgdorferi

Mouse	B. burgdorferi	OspA	OspB	Media (control)
Nontransgenic (control)	7.3 (81,760)	10.8 (120,960)	2.5 (28,000)	0 (11,200)
OspA-transgenic	8.4 (90,720)	2.1 (22,680)	2.8 (30,240)	0 (10,800)
OspB-transgenic	9.8 (80,360)	13.5 (110,700)	0 (7,600)	0 (8,200)

Mice were immunized with 10 μ g of *B. burgdorferi* in CFA. Lymph nodes were harvested after 9 d and stimulated with *B. burgdorferi*, OspA or B, as indicated in the methods section. Responses were measured by the uptake of [³H]thymidine, and the actual counts per minute (cpm) are indicated in parentheses. Stimulation indices (SI) represent the mean experimental cpm divided by the mean control cpm.

antibodies, or OspA T cell responses, correlate with the development of, and may therefore be implicated in the genesis of treatment-resistant chronic Lyme arthritis (7, 8). Furthermore, a cellular immune response to OspA may be involved in the development of Lyme arthritis because T_h1 type T cells can be isolated from the synovial fluid of patients with Lyme arthritis, and the injection of OspA into the joints of rats has been shown to elicit an inflammatory response (10, 11, 30). The murine model of Lyme borreliosis partially mimics human infection, including the development of arthritis and carditis and is therefore a useful system for studying whether responses to specific B. burgdorferi antigens are involved in disease pathogenesis. In this study transgenic mice were generated that expressed OspA or B, and were tolerant to OspA or B respectively since the mice are unable to mount a significant humoral or cellular response to these antigens. The evolution of Lyme borreliosis in these animals, that could no longer elicit a response to OspA

or	B,	was	assessed	i as	a mea	ns of	determ	nining	whether	the
gei	nera	ation	of an Os	pA o	r B spo	ecific i	immune	e respo	onse is ne	ces-
sar	y f	or the	e develoj	omen	t or ev	olutio	n of m	urine o	disease.	

Our studies show that the OspA or B transgenic mice cannot generate a humoral immune response to OspA or B, respectively, and that the ablation of the immune response is antigenspecific. Moreover, antigen-specific cellular immune responses to OspA or B were markedly diminished or ablated in the transgenic mice. OspB transgenic mice did not mount proliferative responses to OspB, whereas OspA transgenic and control mice had similar, moderate cellular responses to OspB. In addition, OspA transgenic mice exhibited markedly decreased proliferative responses to OspA when compared to the OspA responses in OspB transgenic animals or control mice. When OspA or B transgenic mice were, however, challenged with the spirochete, the evolution of Lyme borreliosis, including the recovery of spirochetes from selected murine tissues, the devel-

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		Disease			Isolation of <i>B. burgdorferi</i>				
Mouse	Day	Joints	Severity of arthritis	Heart	Blood	Spleen	Bladder	Culture-positive	
B6 (10⁴)	14	13/28 (16/28)	0.8±0.2 (0.6±0.3)	11/14 (12/14)	6/14 (8/14)	11/14 (7/14)	11/14 (11/14)	13/14 (13/14)	
	30	31/40 (30/40)	1.1±0.3 (1.0±0.2)	16/20 (15/20)	3/20 (7/20)	11/20 (8/20)	13/20 (14/19)	17/20 (16/20)	
	60	17/27 (19/26)	$0.8^{r} \pm 0.1 \ (0.7^{r} \pm 0.2)$	11/13° (9/13°)	5/13 (1/13)	9/13 (5/13)	11/13 (12/13)	12/13 (13/13)	
	120	8/26 (7/26)	$0.5^{r}\pm0.2 \ (0.5^{r}\pm0.1)$	13/13° (12/13°)	1/13 (2/13)	6/13 (5/13)	12/13 (11/13)	13/13 (13/13)	
	180	6/20 (3/20)	$0.5^{r}\pm0.3 (0.5^{r}\pm0.2)$	7/10 ^c (8/10 ^c)	1/10 (2/10)	(2/10) (1/10)	7/10 (8/10)	7/10 (8/10)	
B6 (10 ⁷)	14	3/4 (4/4)	$1.1\pm0.4~(0.9\pm0.2)$	2/2 (2/2)	1/2 (1/2)	1/2 (2/2)	1/2 (1/2)	2/2 (2/2)	
	30	14/16 (12/16)	$0.9 \pm 0.2 (1.0 \pm 0.3)$	7/8 (8/8)	2/8 (1/8)	2/8 (2/8)	8/8 (7/8)	8/8 (8/8)	
	60	10/20 (12/20)	$1.0^{r} \pm 0.3 \ (0.8^{r} \pm 0.2)$	9/10 ^c (10/10 ^c)	3/10 (2/10)	6/10 (5/10)	8/10 (7/10)	9/10 (8/10)	
	90	4/8 (2/8)	$1.0^{r} \pm 0.3 \ (0.9^{r} \pm 0.3)$	4/4 ^c (4/4 ^c)	0/4 (0/4)	2/4 (1/4)	3/4 (4/4)	4/4 (4/4)	
	180	4/20 (3/20)	$0.4^{r}\pm0.2 \ (0.2^{r}\pm0.1)$	9/10 ^c (10/10 ^c)	1/10 (1/10)	1/10 (2/10)	6/10 (7/10)	7/10 (8/10)	
C3H (10 ⁴)	14	14/16 (12/16)	1.7±0.4 (1.7±0.5)	8/8 (8/8)	7/8 (6/8)	6/8 (8/8)	8/8 (8/8)	8/8 (8/8)	
	30	18/26 (20/26)	1.9±0.3 (1.8±0.4)	12/13 (12/13)	4/13 (5/13)	7/13 (10/13)	8/13 (7/13)	11/13 (10/13)	
	60	15/24 (13/20)	$1.0^{r} \pm 0.4 \ (0.9^{r} \pm 0.3)$	12/12 ^c (9/10 ^c)	2/12 (0/10)	5/8 (4/10)	5/12 (7/10)	9/12 (8/10)	
	120	4/24 (4/22)	$0.5^{r}\pm0.1$ ($0.5^{r}\pm0.1$)	10/12 ^c (11/11 ^c)	2/12 (2/11)	6/12 (3/11)	9/12 (6/11)	11/12 (10/11)	
	180	4/18 (1/16)	$0.5^{r}\pm0.2 \ (0.5^{r}\pm0.1)$	8/9 ^c (7/8 ^c)	1/9 (0/8)	2/9 (0/8)	3/9 (5/8)	4/9 (5/8)	
C3H (10 ⁷)	30	4/6 (5/6)	1.3±0.4 (1.0±0.2)	3/3 (3/3)	0/3 (0/3)	0/3 (0/3)	1/3 (3/3)	1/3 (3/3)	
	180	3/18 (4/20)	0.5 ^r ±0.3 (0.3 ^r ±0.2)	9/9 ^c (10/10 ^c)	2/9 (2/10)	1/9 (1/10)	3/9 (5/10)	5/9 (6/10)	

Transgenic mice and nontransgenic littermates (controls) were challenged with 10⁴ or 10⁷ *B. burgdorferi* (indicated as 10⁴ or 10⁷) and killed at selected timepoints between 14 and 180 d. Tissues (blood, spleen and bladder) were cultured in BSK medium for 2 wk and examined by darkfield microscopy for the presence of spirochetes. Mice from which *B. burgdorferi* were isolated from at least one tissue specimen were considered culture-positive. The knees, tibiotarsi and heart were examined microscopically for inflammation. The joint disease represents the tibiotarsi because these joints uniformly had more disease than the knees. Arthritis was graded (mean±standard deviation) on a scale from 0 to 3 in a double-blind fashion according to our published data. ' Resolving disease. Carditis was not graded. ^c Chronic, inactive, lesions. The results of control mice are indicated by parentheses.

Table III. Lyme Borreliosis in OspB-transgenic Mice

	Day	Disease			Isolation of B. burgdorferi					
Mouse		Joints	Severity of arthritis	Heart	Blood	Spleen	Bladder	Culture-positive		
B6 (10 ⁴)	14	2/10 (4/10)	0.5±0.3 (0.5±0.2)	4/5 (4/5)	1/5 (3/5)	4/9 (4/5)	4/5 (4/5)	5/5 (5/5)		
	30	9/18 (14/16)	0.7 ± 0.2 (1.0 ± 0.6)	9/9 (7/8)	1/9 (2/8)	3/9 (5/8)	8/9 (7/8)	9/9 (8/8)		
	60	7/18 (12/14)	$0.9^{r} \pm 0.3 (1.0^{r} \pm 0.4)$	6/9° (7/7°)	0/9 (0/7)	4/9 (4/7)	8/9 (6/7)	9/9 (7/7)		
	120	1/16 (5/18)	$0.5^{t}\pm0.2 \ (0.5^{t}\pm0.1)$	8/8 ^c (9/9 ^c)	0/8 (1/9)	2/8 (1/9)	8/8 (9/9)	8/8 (9/9)		
	180	0/20 (3/16)	$0.3^{r} \pm 0.2 \ (0.4^{r} \pm 0.3)$	10/10° (16/16°)	0/10 (0/8)	(3/10) (3/8)	5/10 (3/8)	7/10 (7/8)		
B6 (10 ⁷)	14	11/16 (12/16)	0.8±0.1 (0.7±0.2)	8/8 (8/8)	5/8 (4/8)	3/8 (6/8)	6/8 (5/8)	8/8 (8/8)		
	30	7/10 (6/10)	0.9 ± 0.2 (1.1 ± 0.3)	5/5 (5/5)	1/5 (2/5)	2/5 (1/5)	4/5 (5/5)	5/5 (5/5)		
	180	1/8 (2/8)	$0.4^{r}\pm0.2 \ (0.2^{r}\pm0.3)$	3/4 ^c (2/4) ^c	0/4 (0/4)	2/4 (3/4)	2/4 (2/4)	3/4 (4/4)		
C3H (10 ⁷)	30	8/8 (8/8)	1.4±0.3 (1.2±0.4)	4/4 (4/4)	2/4 (3/4)	3/4 (3/4)	2/4 (3/4)	4/4 (4/4)		
	180	2/10 (4/10)	$0.2^{r}\pm0.2 (0.5^{r}\pm0.3)$	4/5 ^c (4/5 ^c)	2/5 (0/5)	1/5 (1/5)	1/5 (1/5)	3/5 (2/5)		

Transgenic mice and non-transgenic littermates (controls) were challenged with 10^4 or 10^7 *B. burgdorferi* (indicated as 10^4 or 10^7) and sacrificed at selected timepoints between 14 and 180 d. Tissues (blood, spleen and bladder) were cultured in BSK medium for 2 wk and examined by darkfield microscopy for the presence of spirochetes. Mice from which *B. burgdorferi* were isolated from at least one tissue specimen were considered culture-positive. The joints (tibiotarsi and knees) and heart were examined microscopically for inflammation. The joint disease represents the tibiotarsi, for these joints uniformly had more disease than the knees. Arthritis was graded (mean±standard deviation) on a scale from 0-3 in a double blind fashion according to our published data. 'Resolving lesion or residual scarring. Carditis was not graded; ^c Chronic, inactive lesions. The results of control mice are indicated by parentheses.

opment of arthritis and carditis, and the subsequent resolution of disease remained similar in both the transgenic animals and nontransgenic littermates. In both cases the arthritis was most prominent between 14 and 30 d after the infectious challenge and began to resolve over a period of 1-3 mo. These data suggest that the immune responses to OspA or B is not necessary for the generation of an arthritogenic response in the C3H or B6 mouse. This is consistent with our previous studies showing that immunization with OspA is not sufficient to induce arthritis in mice (29). Indeed, the assessment of the effects of antigen-specific ablation of the OspA or B immune response in animals that are highly-susceptible to disease (C3H) or relatively resistant to disease (B6) strongly indicate that an OspA or B response is not singularly required for the development or resolution of disease.

In this system we have selectively eliminated the murine immune response to OspA or B. It is possible that ablation of both the OspA and B immune responses are necessary to cause an alteration in the genesis of disease. However, if both antigens contributed to the development of a pathogenic immune response, it is likely that a partial reduction in the degree of arthritis would have been apparent in transgenic mice that are tolerant to the individual antigens. Studies with transgenic animals that are tolerant to both OspA and B may help address this issue. Furthermore, it can be suggested that the OspA or B response does not contribute to the pathogenesis of arthritis in C3H or B6 mice—but it can still be argued that these model systems cannot be directly extended to the human illness as the murine model only partially mimics the human infection. Indeed, the murine model most closely resembles acute arthritis and may not accurately depict chronic antibiotic-resistant human Lyme arthritis. Unfortunately, similar studies of the ablation of the OspA or B response in humans are not possible. Nonetheless, in many way the murine models have provided much useful information with regard to the immune response to B. burgdorferi that has been applicable to the human illness, most noticeably with respect to vaccine development and disease pathogenesis (18-20, 23, 33). Indeed, it was initially suggested that the murine and human infections differed in that mice developed an early antibody response to OspA, whereas humans developed antibodies to OspA in the later stages of infection, if at all (6). These initial murine studies used syringe challenge to inoculate the spirochetes, and subsequent studies using tick-mediated transmission in the murine model, show that the murine response to *B. burgdorferi* closely resembles the human response (6, 24). Therefore, in as much as human infection is similar to murine Lyme borreliosis, these data strongly suggest that the OspA or B immune responses are not essential for the genesis of arthritis. This study, however, does not address the issue of the safety or potential limitation of an OspA-based vaccine in humans.

These studies also show that the immune responses to OspA or B are not individually required for the modulation and regression of disease in mice. Several groups have shown that scid mice develop severe persistent arthritis that does not undergo resolution-suggesting that the immune response that is generated during infection plays a role in the control of infection and the resolution of disease (16, 17). The passive transfer of antiserum from mice infected with B. burgdorferi to infected scid mice can result in the modulation and regression of the disease-thereby transforming the course of disease in the scid mouse to the evolution of disease more commonly seen in the immunocompetent animals (S. Barthold, unpublished data). Moreover, arthritis in C3H mice begins to resolve as the animals develop an anti B. burgdorferi response, that-depending on the challenge dose of spirochetes-may or may not contain antibodies to OspA or B (24, 25). Our studies, in OspA- or Btransgenic mice, show that the evolution of disease in the transgenic and nontransgenic littermates is similar, despite the inability of the transgenic mice to mount an antigen-specific response. Moreover, the data further suggests that the murine response to OspA, as in human Lyme borreliosis, occurs in the later stages of infection. This indicates that the immune response to OspA or B is not necessary for the regression of arthritis and carditis, and that the immune response, specifically antibodies, to other B. burgdorferi proteins can modulate disease.

In summary, we have successfully generated transgenic mice that are tolerant to *B. burgdorferi* OspA or B—the animals have a significantly diminished ability to respond to, or lack humoral and cellular immune responses to these specific antigens. We have shown that this ablated response does not alter the genesis of murine Lyme disease, including infection, arthritis and carditis, and suggests that the immune response to OspA or B is not singularly required to causes arthritis or carditis or result in the regression of disease in the *B. burgdorferi* infected mouse.

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