Antibody-Mediated Disease Remission in the Mouse Model of Lyme Borreliosis

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In the mouse model of Lyme borreliosis, the host immune response during infection with *Borrelia burgdorferi* results in the remission of carditis and arthritis, as well as global reduction of spirochete numbers in tissues, without elimination of infection (28). These events were recapitulated by passive transfer of immune serum from infected immunocompetent mice or T-cell-deficient mice to severe combined immunodeficient (SCID) mice. Previous studies have shown that immune serum is reactive against arthritis-related protein (Arp) and that Arp antiserum induces arthritis remission (16). However, although immune serum from T-cell-deficient mice induced disease remission, it was not reactive against Arp, suggesting that antibody to another antigen may be responsible. T-cell-deficient mouse immune serum was reactive to decorin binding protein A (DbpA). Therefore, DbpA antiserum was tested to determine its ability to induce disease remission in SCID mice. Antisera to Arp or DbpA induced both carditis and arthritis remission but did not significantly reduce spirochete numbers in tissues, based upon quantitative *flaB* DNA analysis, nor did treatment affect RNA levels of several genes, including *arp* and *dbpA*. Immunohistochemical labeling of spirochetes in hearts and joints during disease remission induced by adoptive transfer of lymphocytes, passive transfer of immune serum, or passive transfer of DbpA antiserum revealed that such treatment resulted in elimination of spirochetes from heart base and synovium but not vascular walls, tendons, or ligaments. These results suggest that Arp and DbpA antibodies may be active as disease-resolving components in immune serum but antibody against other antigens may be involved in reductions of spirochetes in tissues.

Lyme borreliosis, caused by tick-borne *Borrelia burgdorferi*, has numerous clinical manifestations in humans, including arthritis and carditis, which undergo remission and periodic bouts of exacerbation over the course of months to years of persistent infection (42). Laboratory mice inoculated with *B. burgdorferi* also develop arthritis and carditis, which evolve over the course of three weeks and then undergo immune-mediated remission and recurrence during persistent infection (1, 5, 8). In the mouse model, remission of disease requires adaptive immunity of the infected host, since it does not occur in infected severe combined immunodeficient (SCID) mice (9, 39, 40, 49). Disease remission in the mouse model is critically dependent upon the humoral immune response. B-cell-deficient mice develop progressively severe arthritis and carditis when infected with *B. burgdorferi*, whereas T-cell-deficient mice undergo disease remission (36). Passive transfer of immune sera from actively infected immunocompetent mice into infected SCID mice results in disease remission but does not eliminate infection (4, 6).

Based upon these findings, which implicate antibody in disease remission, we have screened a *B. burgdorferi* λ ZAP II DNA genomic expression library with sera from actively infected mice (immune sera) to identify immunoreactive *B. burgdorferi* gene products that might be mediators of these critical host immune responses. One gene product (homologous to that of *B. burgdorferi* B31 BBF01) is arthritis-related protein

(Arp). Passive transfer of Arp antiserum into infected SCID mice was shown to induce arthritis remission (16). However, this does not explain how arthritis resolves in T-cell-deficient mice, since immune serum from actively infected T-cell-deficient mice does not react against Arp (present study). These findings suggest that another *B. burgdorferi* protein, particularly one that elicits a T-cell-independent antibody response, might be involved in disease remission. A strong candidate is decorin binding protein A (DbpA), to which infected mice undergo seroconversion within 2 weeks of infection (17), and it is one of only a few *B. burgdorferi* antigens that is reactive with antibody in serum from infected T-cell-deficient mice (35).

In the present study, we determined that passive transfer of immune serum from actively infected immunocompetent or T-cell-deficient mice to infected SCID mice induced remission of both arthritis and carditis, as well as global reductions of spirochetes in tissues. Arp or DbpA antiserum, on the other hand, also induced disease remission but did not significantly reduce spirochete numbers in tissues. This prompted immunohistochemical analysis of hearts and joints during disease remission; this analysis revealed selective elimination of spirochetes from specific tissue sites and sparing of spirochetes in other sites.

MATERIALS AND METHODS

Mice. Specific-pathogen-free, 3- to 5-week-old C3H/HeN (C3H) and C3H/Smn. C1cr-Prdc^{scid} (C3H-scid) mice were purchased from Frederick Cancer Research Center (Frederick, MD) and Harlan Sprague-Dawley, Inc. (Indianapolis, IN), respectively. C57BL/6J (B6) and T-cell-deficient, B6.129-Tcr^{gmlMom}Tcr^{gmlMom} (B6-Tcr β /8) mice were purchased from The Jackson Laboratory, Bar Harbor, ME. Mice were killed by carbon dioxide narcosis, followed by cardiac exsanguination.

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***Borrelia burgdorferi*.** A clonal isolate of *B. burgdorferi* strain N40 (cN40) was cultured in modified Barbour-Stoenner-Kelly II medium (3). Mice were infected by intradermal inoculation of either 10^3 or 10^4 mid-log-phase spirochetes in 0.1 ml of Barbour-Stoenner-Kelly II medium on the dorsal thoracic midline. Infection status of all mice was confirmed at necropsy by culture of urinary bladder, as previously described (5).

Histopathology. Rear limbs and hearts were fixed in neutral buffered formalin, pH 7.2. Bones were demineralized and sections were processed and stained with hematoxylin and eosin by routine histologic methods. The prevalence of arthritis in each mouse was determined by examination of four joints (both knees and tibiotarsi). Tibiotarsal arthritis severity was scored on a scale of 0 (negative) to 3 (severe), as described previously (4). The arthritis score for each mouse was determined as the average of the scores of both tibiotarsi when both legs were examined, and the mean score $\pm$ standard deviation (SD) was calculated for each treatment group. Sagittal sections through the heart, including the great vessels at the heart base, were examined for active inflammation, characterized by transmural infiltration of neutrophils in the aorta, pulmonary artery, and/or coronary artery and infiltration of surrounding connective tissue with macrophages, as described previously (1, 9). Carditis was scored with a scale of 0 (no active inflammation), 1 (mild active inflammation), 2 (moderate active inflammation), or 3 (severe active inflammation). Tissue sections were blindly examined without knowledge of treatment.

Immunohistochemistry. Formalin-fixed and demineralized (rear legs), paraffin-embedded tissues were sectioned at 5 μ m, and then incubated in 3% H_2O_2 for 20 min to block endogenous peroxidase, washed with phosphate-buffered saline, treated with protease (0.5 mg/ml) (Sigma-Aldrich, Milwaukee, WI) for 10 min, washed again, and then blocked with Power block (BioGenex, San Ramon, CA) for 15 min. Sections were incubated for 30 min with rabbit immune serum diluted 1:1,000. Following incubation with the primary rabbit antibody, slides were incubated for 20 min with biotinylated goat anti-rabbit immunoglobulin G (Vector Laboratories, Burlingame, CA) diluted 1:500, washed with phosphate-buffered saline, and then incubated with peroxidase-conjugated streptavidin (Vector) for 20 min. For visualization of spirochetes, the chromogen 3,3'-diaminobenzidine tetrahydrochloride in 0.24% H_2O_2 was used. Sections were counterstained with Mayer's hematoxylin and mounted with coverslips.

Recombinant protein preparation. Fifteen *B. burgdorferi* N40-specific recombinant proteins and *B. burgdorferi* N40 lysates were utilized as antigens for serology. Their molecular weight, laboratory designation, relationship to published *B. burgdorferi* B31 genes (21), and *B. burgdorferi* N40-specific GenBank accession numbers are summarized in Table 1. Proteins for inclusion were selected from a collection of 46 immunoreactive clones, as described previously (16). Each gene was PCR amplified and cloned in frame with the glutathione *S*-transferase gene into pMX, a pGEX-2T vector (Pharmacia, Piscataway, NJ) with a modified polylinker (41). The PCR-amplified DNA sequences of the recombinant DNA were confirmed by sequence comparison with the original inserts. Recombinant proteins were purified on glutathione columns and freed of their glutathione *S*-transferase fusion partners by thrombin cleavage as described previously (18).

Enzyme-linked immunosorbent assay. One hundred microliters of 1 μ g/ml *B. burgdorferi* N40 lysates or recombinant protein in carbonate coating buffer (pH 9.6) was plated in 96-well plates, as described previously (17). Duplicate samples of each mouse serum were tested, including uninfected normal mouse serum as a control. Sera were serially diluted 10-fold, with a starting dilution of 1:100. The secondary antibody was alkaline phosphatase-conjugated goat anti-mouse immunoglobulin G diluted at 1:5,000 (Jackson Immuno-Research Laboratories Inc., West Grove, PA). Optical density values were read on a kinetic microplate reader (Molecular Devices, Sunnyvale, CA). Values were subtracted from background reactivity against normal mouse serum (optical density at 405 nm).

Immune sera and hyperimmune antisera. Immune sera were generated by intradermal inoculation of mice with 10^3 or 10^4 *B. burgdorferi* cN40. At 60 or 90 days after inoculation, mice were exsanguinated and infection was verified by culture. Hyperimmune antisera against recombinant proteins were generated by subcutaneous immunization of C3H mice with 20 μ g protein emulsified in complete Freund's adjuvant (0.1 ml total volume) followed by two boosts at 2-week intervals with 10 μ g protein emulsified in incomplete Freund's adjuvant. Antibody titer to each recombinant protein was verified by enzyme-linked immunosorbent assay at a dilution of at least 1:100,000. Rabbit immune serum was prepared by intradermal inoculation of 10^6 spirochetes into multiple sites on the shaved backs of two New Zealand White rabbits. Serum was harvested at necropsy on day 84.

Quantitative analysis of *B. burgdorferi* DNA and RNA. For quantitative analysis of DNA or cDNA extracted from tissues, real-time PCR was standardized

TABLE 1. Reactivity of 90-day immune serum from infected B6 mice versus T-cell-deficient B6-*Tcr β / δ* null mice against selected immunoreactive *B. burgdorferi* antigens^a

Protein ^b	B31 gene	N40 GenBank accession no.	Reciprocal dilution ^c	
			B6 serum	B6- <i>Tcrβ/δ</i> KO serum
<i>B. burgdorferi</i> cN40 lysate			1:200,000	1:2,700
P24-13	BB0015	AF102697	<1:100	<1:100
OspA	BBA15	M57248	1:1,000	1:1,000
OspC	BBB19	AY275221	1:1,000	<1:100
BmpA	BB0383	AF288609	1:10,000	<1:100
Arp (ErpT)	BBF01	AF050212	1:100,000	<1:100
DbpA	BBA24	BBU63932	1:1,000,000	1:10,000
P12	None assigned ^d	AF011454	1:100,000	1:1,000
P19-23	None assigned ^d	AF288474	1:1,000	<1:100
P20-13	BB0014	AF102696	1:1,000	<1:100
P23-T2	BBQ38	AY942708	1:1,000	<1:100
P29-23	BB0811	AF288475	1:1,000	<1:100
P31	BBK46	AF092839	1:1,000	<1:100
P37-42	BBK47	AF035553	1:1,000	<1:100
P45-13	BBA57	AF102699	1:10,000	<1:100
P61	BB0329	AF050213	1:10,000	1:1,000

^a Mice were inoculated with 10^4 *B. burgdorferi* cN40, a dose that elicits a low response to OspA.

^b *B. burgdorferi* N40 homologues of B31 genes or laboratory designation (the numbers following "P" indicate molecular weight, and numbers following hyphens indicate laboratory clone numbers). Unpublished gene sequences for underlined proteins were submitted to GenBank by S. Feng.

^c Reciprocal dilution, starting with a dilution of 1:100.

^d No homologous sequence in the B31 genome.

and optimized for each gene. Each assay was performed in triplicate, with positive and negative control samples, as previously described (28, 29). At necropsy, tissue samples were collected and immediately weighed, snap-frozen in liquid nitrogen, pulverized and homogenized, and then split equally for DNA or RNA extraction. DNA was extracted from samples using DNeasy tissue kits according to the manufacturer's instruction for tissues (QIAGEN, Valencia, CA). Three oligonucleotides, two primers and a probe, for the *B. burgdorferi* *flaB* gene were designed and utilized, as described previously (28). Primers for the mouse cytoplasmic β -actin gene (GenBank accession no. M12481) were selected using Primer Express software (PE Biosystems, Foster City, CA). Primers Act-256F (5'-CCTAAGGCCAACCCTGAAAA-3') and Act-393R (5'-GTCACCGAGTCCATCACAAT-3') were synthesized to amplify a 138-bp fragment of a β -actin DNA. The internal oligonucleotide probe represented Act-302P (5'-TCAACACCCAGCCATGTACGTAGCC-3'). Total RNA was purified with RNeasy mini kits, according to the manufacturer's instructions (QIAGEN). Samples were homogenized with a QIAshredder and then treated with RNase-free DNase I prior to elution. The concentration and purity of extracted RNA were determined as described previously (29). Published primer sets for *dbpA*, *arp*, *ospC*, *p37-42*, and the fibronectin binding protein gene (*fbp*) were utilized (28), based upon *B. burgdorferi* N40-specific sequences for each gene, determined from clones derived from the λ ZAP II genomic library, as described previously (16).

In one experiment, the copy number of *flaB* DNA was expressed per copy number of β -actin DNA, since the mouse cytoplasmic β -actin gene, which is extremely conserved (45), has a single copy in C3H mice (15). In spite of these considerations, quantification of gene copies for tissues with different cellular composition and various degrees of inflammation (and therefore cellularity) was deemed to be more accurately analyzed based upon copy number per mg of tissue weight, as described previously (28).

Statistics. Statistical comparisons between different mouse groups and time points were made using Student's unpaired *t* test. Multiple-comparison analyses were made with one-way analysis of variance, followed by a least-difference post hoc test (StatView, PowerPC version, SAS Institute Inc., Cary, NC). Calculated *P* values lower than 0.05 were considered significant.

Nucleotide sequence accession numbers. The GenBank accession numbers for the sequences presented in this paper are listed in Table 1.

FIG. 1. *Borrelia burgdorferi* *flaB* DNA per mg tissue weight (means $\pm$ standard deviations) in tissues of C3H-*scid* mice treated with immune serum from C3H mice infected for 90 days (black bars) compared to mice treated with normal C3H mouse serum (gray bars) (*, $P \leq 0.05$).

RESULTS

Induction of disease remission and tissue spirochete reduction with immune serum. Recent studies compared *B. burgdorferi* population dynamics and selected gene transcription in C3H and C3H-*scid* mice and demonstrated that the evolution of the acquired immune response in C3H mice resulted in carditis and arthritis remission, as well as global reductions of spirochete populations in a variety of tissues (28). In an effort to demonstrate that these events can be recapitulated by passive immunization of infected SCID mice with immune serum, 10 C3H-*scid* mice were inoculated with *B. burgdorferi*, and then groups of five mice were treated with either 0.3 ml C3H mouse immune serum after 90 days of infection (90-day immune serum) or 0.3 ml normal mouse serum on days 12, 18, and 24 after *B. burgdorferi* inoculation. Mice were necropsied on day 28. Infection of all mice was confirmed by culture of urinary bladder. The right knee and tibiotarsus were examined for arthritis, and the ear, heart base, ventricular muscle, left tibiotarsus, and quadriceps muscle were collected for quantitative *flaB* DNA analysis. In addition, heart base, ventricular muscle, tibiotarsus, and quadriceps muscle were processed for RNA levels of *flaB*, *ospC*, *dbpA*, *arp*, *p37-42*, and *fbp*.

All C3H-*scid* mice treated with normal mouse serum had arthritis in both the knee and tibiotarsus (mean tibiotarsal score $\pm$ SD, 2.0 ± 0), and all C3H-*scid* mice treated with 90-day immune serum had no arthritis (mean tibiotarsal score, 0). Spirochete numbers were reduced in all tissues from mice treated with immune serum compared to those of mice treated with normal mouse serum (Fig. 1). RNA levels of *ospC*, *dbpA*, *arp*, *p37-42*, and *fbp* were generally reduced in tissues of mice treated with immune serum, commensurate with reductions in *flaB* DNA (data not shown). These results confirm that immune serum results in disease remission and reduction of spirochetes in various tissues, with concomitant reduction of

RNA levels, similar to events in untreated infected C3H mice versus C3H-*scid* mice (28).

Induction of disease remission and tissue spirochete reduction with T-cell-deficient mouse immune serum. We previously found that both arthritis and carditis undergo immune-mediated remission in B6-*Tcr β / δ* null (T-cell-deficient) mice, but passive transfer of immune serum from these mice did not induce arthritis or carditis remission in infected C3H-*scid* mice (35). This result did not fit with the effects of immune serum from fully immunocompetent mice (described above) and may have been influenced by treatment with insufficient amounts of serum. We therefore reexamined this result by using a higher dose of B6-*Tcr β / δ* immune serum that was more equivalent to the total amount of serum in a mouse. Twelve C3H-*scid* mice were inoculated with *B. burgdorferi*. On days 12, 18, and 24 after inoculation, groups of four C3H-*scid* mice were passively immunized with 0.4 ml of 90-day immune serum from immunocompetent B6 mice (positive control), 0.75 ml of 90-day immune serum from T-cell-deficient B6-*Tcr β / δ* mice, or 0.4 ml of normal B6 mouse serum (negative control). Mice were necropsied on day 28, and the infection status of each mouse was confirmed by culture. Both knees and tibiotarsal joints were examined for inflammation, and ear tissue was collected for quantitative analysis of *flaB* DNA.

Infected C3H-*scid* mice treated with B6 immune serum had no arthritis and no carditis (complete resolution). All infected C3H-*scid* mice treated with normal B6 mouse serum had arthritis in both knee and both tibiotarsal joints (mean arthritis prevalence [joints affected] $\pm$ SD, 4.0 ± 0 ; mean tibiotarsal arthritis severity score $\pm$ SD, 2.5 ± 0.4). All four C3H-*scid* mice also had acute carditis (severity score $\pm$ SD, 2.0 ± 0). In contrast, only one of the four C3H-*scid* mice treated with B6-*Tcr β / δ* mouse immune serum had arthritis in the knee joints (mean prevalence $\pm$ SD, 2.0 ± 1.4 joints affected), and the mean tibiotarsal severity score $\pm$ SD was 1.4 ± 0.8 . Furthermore, carditis was less severe in C3H-*scid* mice treated with B6-*Tcr β / δ* mouse immune serum (severity score $\pm$ SD of 1.0 ± 0) than in C3H-*scid* mice treated with normal B6 mouse serum. Thus, this experiment showed that like immune serum from immunocompetent mice, immune serum from T-cell-deficient mice also contains antibody that induces both carditis and arthritis remission.

Quantitative analysis of ear tissue from these mice revealed a reduction of spirochete numbers (based upon copy numbers of *flaB* DNA per 10^6 copy numbers of β -actin DNA) in C3H-*scid* mice treated with B6 mouse immune serum ($4.5 \times 10^3 \pm 3.1 \times 10^3$ *flaB* copy numbers) and B6-*Tcr β / δ* mouse immune serum ($1.9 \times 10^4 \pm 0.7 \times 10^4$ *flaB* copy numbers) compared to C3H-*scid* mice treated with normal mouse serum ($2.6 \times 10^6 \pm 0.02 \times 10^6$ *flaB* copy numbers). B6 mouse immune serum caused a greater reduction of *flaB* copy numbers compared to B6-*Tcr β / δ* mouse immune serum ($P = 0.0062$) and B6 normal mouse serum ($P = 0.032$). B6-*Tcr β / δ* mouse immune serum caused an intermediate level of reduction in *flaB* copy numbers compared to normal mouse serum ($P = 0.244$). Although B6-*Tcr β / δ* mouse immune serum did not cause a statistically significant reduction, the trend was apparent.

Thus, immune sera from immunocompetent B6 mice as well as B6-*Tcr β / δ* mice were capable of inducing remission of both carditis and arthritis, as well as reducing spirochete numbers in tissue when passively transferred to infected C3H-*scid* mice.

TABLE 2. Arthritis severity in C3H-*scid* mice treated with hyperimmune sera against selected recombinant proteins compared with C3H-*scid* mice with no serum treatment

Antiserum	Mean tibiotarsal arthritis score $\pm$ SD
DbpA.....	1.2 $\pm$ 0.4
Arp.....	1.3 $\pm$ 0.5
OspA.....	2.4 $\pm$ 0.5
P37-42.....	2.6 $\pm$ 0.9
None	2.8 $\pm$ 0.4

Because of the expected lower titer of antibody in immune serum from B6-*Tcr β / δ* mice (35), the effect of B6-*Tcr β / δ* immune serum was not as potent as B6 mouse immune serum, despite the larger volume of serum passively transferred.

Reactivity of B6 versus B6-*Tcr β / δ* mouse immune sera against a panel of immunoreactive *B. burgdorferi* recombinant proteins. We have accrued a collection of *B. burgdorferi* N40 recombinant proteins that have been derived from screening a λ ZAP II *B. burgdorferi* N40 genomic expression library with immune sera from infected mice (16). This effort was undertaken because *B. burgdorferi* lysates, derived from cultured spirochetes, do not necessarily reflect the antigenic profile of *B. burgdorferi* in vivo. Indeed, several proteins have been shown to be exclusively or relatively expressed in vivo (11, 12, 19, 44, 46). Immune serum from B6 mice reacted strongly to *B. burgdorferi* lysates and to all of the recombinant proteins, excepting P24-13 (negative control) (Table 1). The highest titer reactivity was to DbpA. In contrast, immune serum from B6-*Tcr β / δ* mice reacted to *B. burgdorferi* lysates, OspA, DbpA, P12, and P61 but not the other proteins, including Arp. Low-titer reactivity to OspA was apparent in both types of immune sera, which reflects the presence of OspA in the inoculum (7). Notably, immune serum from B6-*Tcr β / δ* mice reacted particularly strongly to DbpA relative to all other antigens. These results reinforced our interest in further examination of DbpA as an alternate candidate antigen for antibody-mediated disease remission.

Disease remission in C3H-*scid* mice treated with DbpA and Arp antisera. The effect of DbpA antiserum, compared to Arp antiserum, P37-42 antiserum, or no serum treatment, on arthritis remission and spirochete population dynamics was next determined for C3H-*scid* mice. Arp antiserum was used as a positive-effect control and P37-42 antiserum was used as a negative control based upon previous findings (16). Twenty C3H-*scid* mice were inoculated with *B. burgdorferi*, and then groups of 5 mice were administered 0.3 ml of DbpA, Arp, or P37-42 antisera on days 12, 18, and 24 after inoculation. The fourth group of five mice received no treatment. Arthritis severity was scored at necropsy on day 28. The effect of treatment on spirochete numbers and gene expression in tissues was assessed in heart base, heart ventricular muscle, left tibiotarsus, and quadriceps muscle. These tissues were selected because heart base and tibiotarsus are sites with inflammation, whereas heart ventricular muscle and quadriceps muscle are tissues that are infected, but with minimal inflammation or no inflammation in C3H-*scid* mice (9, 30). Tissue processing for DNA/RNA extraction precluded examination of heart base for

TABLE 3. Prevalence of arthritis and carditis and severity of arthritis in C3H-*scid* mice passively immunized with hyperimmune sera against DbpA, Arp, or OspA

Antiserum	Carditis prevalence	Mean carditis score $\pm$ SD	Arthritis prevalence ^a	Mean tibiotarsal arthritis score $\pm$ SD
DbpA	2/5	0.4 $\pm$ 0.5	6/10	0.8 $\pm$ 0.4
Arp	0/5	0	9/10	1.4 $\pm$ 0.5
OspA	5/5	2.0 $\pm$ 0	10/10	2.8 $\pm$ 0.4

^a Number of joints with inflammation/total number of joints examined. One knee and one tibiotarsus were examined from each of five mice per group.

carditis. Arthritis severity was scored on the right tibiotarsus, and the left tibiotarsus was processed for quantitative DNA/RNA analysis.

Tibiotarsal arthritis was less severe in infected C3H-*scid* mice that received either DbpA or Arp antiserum compared to C3H-*scid* mice that received no serum or P37-42 antiserum (Table 2). Treatment, regardless of group, did not appreciably affect spirochete numbers in tissues (based upon *flaB* DNA copy numbers per mg tissue) (data not shown). Furthermore, there were no differences in RNA levels of *flaB*, *ospC*, *dbpA*, or *arp* in heart base, tibiotarsus, ventricular muscle, or quadriceps muscle among treatment groups (data not shown). In particular, DbpA and Arp antisera, despite their effect upon arthritis and carditis, did not significantly influence RNA levels of either their corresponding gene or other genes.

The previous experiment, which found arthritis remission in mice treated with DbpA and Arp antisera, did not evaluate carditis. In an effort to confirm the effects of DbpA antiserum on arthritis and to evaluate its effect on carditis, 15 C3H-*scid* mice were inoculated with *B. burgdorferi* and then groups of five mice were treated with 0.3 ml DbpA or Arp (positive control) antiserum. In this experiment, we used OspA antiserum as a negative control. Mice were passively immunized on days 12, 18, and 24 after infection. Mice were necropsied on day 28, and infection was confirmed by urinary bladder culture. The right knee and tibiotarsus were evaluated for arthritis and hearts were evaluated for carditis. Heart base, ventricular muscle, left tibiotarsus, quadriceps muscle, and ear were processed for DNA/RNA analysis. In order to evaluate the histology of carditis and nucleic acid in heart samples, the heart was bisected in a sagittal plane with a razor blade.

Treatment of mice with either DbpA or Arp antisera resulted in both arthritis and carditis remission compared to mice treated with OspA antiserum (Table 3). Arthritis was present in fewer joints and was less severe in DbpA and Arp antiserum-treated C3H-*scid* mice. Although joints in these mice were scored as positive, most joints had only minimal inflammation, and the cellular character of the arthritis suggested resolution with few if any neutrophilic leukocytes or fibrin compared to OspA-antiserum-treated mice. These findings demonstrate that treatment of C3H-*scid* mice with either DbpA or Arp antiserum results in resolution of not only arthritis but also carditis.

As described previously, spirochete population dynamics were not significantly altered in mice treated with DbpA or Arp antiserum, compared to OspA antiserum in heart base (Fig. 2), tibiotarsus (Fig. 3), or ventricular or quadriceps muscle (data not shown). Although there was a slight reduction of

FIG. 2. *Borrelia burgdorferi flaB* DNA and *flaB*, *dbpA*, or *arp* cDNA per mg tissue weight (means $\pm$ standard deviations) in hearts of C3H-scid mice treated with antiserum against DbpA, Arp, or OspA.

FIG. 3. *Borrelia burgdorferi flaB* DNA and *flaB*, *dbpA*, or *arp* cDNA per mg tissue weight (means $\pm$ standard deviations) in tibiotarsal joints of C3H-scid mice treated with antiserum against DbpA, Arp, or OspA.

flaB, *dbpA*, and *arp* transcription, commensurate with a slight reduction in *flaB* DNA copies, in mice treated with Arp antiserum, there was no significant difference in transcription of *flaB*, *dbpA*, or *arp* compared to transcription in OspA antiserum-treated mice.

Immunohistochemical labeling of spirochetes in tissues undergoing disease remission. The series of experiments described above demonstrated that immune sera from infected mice contain antibodies that induced remission of both carditis and arthritis and induced global reductions of spirochetes in tissues. In contrast, both Arp and DbpA antisera induced disease remission but without apparent reductions of spirochetes in tissues, including hearts and joints. These seemingly disparate results might suggest that disease remission may be effected by antibody directed against different antigens than those involved in antibody-mediated reduction of spirochetes in tissues. However, the results fail to explain how Arp or DbpA antiserum (or antibody in immune serum) can induce disease remission without elimination of spirochetes from tissues and without apparent effects upon gene transcription.

Evaluation of spirochete population dynamics in tissues in the experiments described above was based upon quantitative analysis of spirochetes in tissue homogenates. This approach fails to measure effects of antisera upon spirochetes in specific tissue types or sites within hearts and joints. Therefore, we examined the distribution of spirochetes within heart and joint tissues of mice that were undergoing immune-mediated disease remission. To accomplish this, we processed three types of mouse tissue for immunohistochemical labeling of spirochetes

during disease remission. We first examined archival tissue from a previous study (30) in which infected C3H-scid mice were immunologically reconstituted at 2 weeks of infection by intravenous adoptive transfer of 2×10^7 splenocytes and 1×10^7 lymph node cells from naïve C3H mice. At 4 and 6 weeks after adoptive transfer (6 and 8 weeks after infection), mice were necropsied. Tissues from groups of five mice at each interval were compared to tissues from groups of five infected C3H-scid mice that were not reconstituted but that were infected at the same intervals. In addition, tissues from five C3H-scid mice treated with 90-day immune serum and five C3H-scid mice treated with normal mouse serum (experiment described above); and five C3H-scid mice treated with DbpA antiserum and five C3H-scid mice treated with OspA antiserum (experiment described above) were processed and examined blindly for presence and patterns of spirochete distribution in tissue.

Findings were similar for tissues of mice from all three experiments. In joint tissues of control infected C3H-scid mice, spirochetes were abundant within the proliferating synovium (Fig. 4A). Adjacent tendons and ligaments were often not infected or contained small numbers of single organisms (Fig. 4B). In contrast, synovial tissues of C3H-scid mice undergoing disease remission were generally devoid of spirochetes (Fig. 4C), but spirochetes were abundant within adjacent tendons and ligaments (Fig. 4D). In contrast to tendons and ligaments of control mice, in which no spirochetes or single spirochetes were found, tendons and ligaments of treated mice often contained clusters of organisms. Distribution of spirochetes tended to

FIG. 4. Immunohistochemical labeling of *B. burgdorferi* in tibiotarsal synovium (A and C) and tendons (B and D) in SCID mice given no treatment (A and B) or adoptive transfer of lymphocytes (C and D). Spirochetes are cleared from synovium (C) but persist and increase in tendons (D) in immune mice.

be multifocal. A similar phenomenon was apparent in hearts. Hearts of control infected C3H-*scid* mice contained readily visible spirochetes in the loose connective tissue of the heart base. In treated mice, spirochetes appeared to be eliminated from this site but became abundant within the adjacent aortic wall near the aortic valve (Fig. 5).

These morphological impressions could not be quantified, but the phenomena were consistent regardless of treatment (adoptive transfer, immune serum, or DbpA antiserum). They provide visual evidence that explains the population dynamic results derived from tissue homogenates. Although there is no net loss of spirochete numbers with treatment, antibody appeared to eliminate spirochetes from the inflamed synovium and loose connective tissue at the heart base, but it spared and perhaps stimulated spirochetes in relatively avascular, highly collagenous tendons, ligaments, and aortic wall.

DISCUSSION

The mouse model has allowed definition of measurable outcomes of the immune response during persistent *B. burgdorferi* infection, including disease remission and reduction of spirochete numbers in tissues, both of which are influenced by the humoral immune response. In the absence of antibody, infection of SCID mice, B6-*Rag1* null mice, and B6-*Igh6* null (B-cell-deficient) mice results in disease progression (9, 35, 36, 39, 40, 50). In contrast, T-cell-deficient B6-*Tcrβ*/δ null mice develop a disease course that is reminiscent of that of fully immunocompetent mice, including disease remission (35, 36). Thus, B cells are both necessary and sufficient to elicit disease remission. Furthermore, passive transfer of immune serum from infected immunocompetent mice to infected SCID mice results in arthritis remission (4, 6). The current study extends these observations by demonstrating that immune serum from

FIG. 5. Immunohistochemical labeling of *B. burgdorferi* in the heart of a SCID mouse following adoptive transfer of lymphocytes. Spirochetes have been eliminated from the loose connective tissue of the heart base (bottom of figure) but are abundant in the adjacent aortic wall.

actively infected mice, including immune serum from T-cell-deficient mice, induces not only arthritis remission but also carditis remission. In addition, immune serum also reduces the spirochete burdens in various tissues when passively transferred to SCID mice. Thus, the effects of passively transferred immune serum into infected SCID mice recapitulate events that take place in persistently infected immunocompetent mice (28).

In a quest to identify antigens that are responsible for disease-resolving antibody during infection, we have screened a *B. burgdorferi* genomic expression library with immune serum from infected immunocompetent mice. Extensive searching and testing of various antisera generated against immunoreactive recombinant proteins derived from the library screening incriminated a 37-kDa lipoprotein, which we termed arthritis-related protein (Arp). Passive transfer of antisera generated against Arp to infected SCID mice resulted in remission of arthritis without eliminating infection (16). The current study extends these results to show that Arp antiserum also induces carditis remission. Arp is the full-length version of a truncated gene product that was previously named ErpT (20), but Arp is not an E-related protein (Erp) paralogue, has minimal amino acid similarity to other Erp proteins, is not encoded on cp32, and does not fit prevailing definitions of an Erp protein (43). Despite its name, Arp is apparently not the only antigen that is responsible for inducing disease-resolving antibody responses during infection. A recent study of human Lyme borreliosis

patients found no correlation between antibody reactivity to Arp and disease status (38). More specifically, examination of the antibody reactivity profile of immune serum from T-cell-deficient mice, which we have shown to induce disease remission and spirochete reductions in tissues, revealed a very limited repertoire of T-cell-independent antigens on immunoblots of *B. burgdorferi* lysates (35). In the current study, we confirmed and extended our previous observation that DbpA is a major antigen recognized during infection of T-cell-deficient mice but that Arp is not. DbpA has been shown previously to elicit active and passive protective immunity against syringe challenge with cultured spirochetes (17, 26, 27) but not against tick-borne infection (25). Another candidate protein that must be considered as a protein that may elicit disease-resolving and spirochete-reducing antibody is OspC. OspC has been shown to induce protective immunity against syringe challenge of some *B. burgdorferi* isolates (10, 34, 37), and treatment of SCID mice infected with *B. burgdorferi* ZS7 with OspC antiserum resulted in not only disease remission but also complete resolution of infection (48, 49). In our studies using mice infected or challenged with *B. burgdorferi* N40, we have not shown either protective or disease-resolving effects of OspC antiserum (6). Furthermore, evaluation of immune sera from T-cell-deficient mice for reactivity against recombinant proteins in the current study did not implicate OspC as a candidate.

Collectively, these observations warranted a more thorough analysis of DbpA as an antigenic target involved in generating

antibodies that induce disease remission and reduction of spirochetes in tissues during infection. Our studies demonstrated that, like Arp antiserum, DbpA antiserum induced both carditis and arthritis remission, but neither antiserum induced reductions of spirochetes in tissues, including hearts and joints, and did not influence gene expression, including *arp* or *dbpA*. These findings did not match the effect of passive transfer of immune serum from actively infected mice into infected SCID mice, which induces both disease remission and reductions of spirochetes in all tissues, including hearts and joints. Thus, immune serum contains factors that elicit several biologically significant events that are measurable by passive transfer, including protective immunity (against syringe challenge), disease-resolving immunity, and global reductions of spirochetes in tissues. We have probed a genomic expression library with immune serum and thereby incriminated two immunoreactive proteins, Arp and DbpA, which elicit protein-specific antibody responses that possess passive protective and disease-resolving activity. Notably, antisera against these specific proteins induce differential effects. For example, Arp antiserum is not protective but induces disease remission (16), and DbpA antiserum is both protective (17, 26) and disease resolving, but neither antiserum induces reductions of spirochetes in tissues. This suggests that antibody (or some nonimmunoglobulin factor) that is present in immune serum is responsible for reducing spirochete burdens in tissues, but that antibody is not likely to be against DbpA or Arp. The specificity of the spirochete-reducing antibody response, if indeed it is antibody, was not within the scope of the present study, but the current findings clearly show that disease remission is not elicited by the same mechanism or same antigenic target as spirochete reductions in tissues. It is likely that spirochete reductions in tissues is also an antibody-mediated event, but that remains to be determined.

The most intriguing aspect of the current study was the demonstration of disease resolution by both Arp and DbpA antisera without reduction of spirochetes in homogenates of various tissues, including hearts and joints, and without apparent effect upon gene transcription, including *arp* and *dbpA*. When we specifically examined the distribution of spirochetes in hearts and joints undergoing immune-mediated disease remission by immunohistochemistry, we found that adoptive transfer of lymphocytes, passive transfer of immune serum, and passive transfer of DbpA antiserum all resulted in elimination of spirochetes from heart base tissue and synovium, but spirochetes continued to be present in vessel walls, tendons, and ligaments. In addition, such treatment actually appeared to stimulate either relocation of spirochetes or replication of spirochetes in these highly collagenous tissues. *Borrelia burgdorferi* is an extracellular pathogen with strong affinity for collagenous tissue. It binds strongly to components of the extracellular matrix and connective tissue, including decorin (23, 24), plasminogen (14, 31), fibronectin (22), glycosaminoglycans (heparan sulfate and dermatan sulfate) (32), integrins (13), and glycosphingolipids (2). A recent publication demonstrated that spirochetes specifically bind to native type I collagen, and the interaction actually stimulates replication and formation of microcolonies within collagen matrices. Notably, the stimulatory effect could be induced with native collagen alone, in the absence of decorin (47). The micro-

colony formation observed in that study parallels the morphological evidence found in the present study, in which apparent clusters or colonies of spirochetes arose in collagenous tissue following immune-mediated disease remission. Another study also provides support for our findings (33). In that study, collagenous tissue was shown to be preferentially colonized in the immune phase of infection in the mouse model, with clearance of spirochetes from noncollagenous tissues. Infection of decorin-deficient mice resulted in population dynamics in various tissues similar to those of wild-type mice in the preimmune phase of infection, but spirochetes did not benefit from the protective niche of collagenous tissue in decorin-deficient mice during the immune phase of infection.

In summary, the current study provides new insight into the host response to *B. burgdorferi* during infection, the specificity of the immune response, and the mechanisms by which spirochetes respond to that immune response. The similar effects observed with Arp and DbpA antisera prompt further investigation of both proteins in the pathogenesis of Lyme borreliosis, including mechanisms of persistence and evasion of host immunity.

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