Borrelia burgdorferi P35 and P37 Proteins, Expressed In Vivo, Elicit Protective Immunity

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Summary

p35 and p37 are Borrelia burgdorferi genes encoding 35 and 37 kDa proteins. The gene products were identified by differential screening of a B. burgdorferi expression library with sera from B. burgdorferi infected and B. burgdorferi–hyperimmunized mice. Northern blot and RT-PCR analyses confirmed that these genes were selectively expressed in vivo. ELISA, using P35 and P37, showed that infected mice (5 of 5, 100%) and patients (31 of 43, 72%) with Lyme borreliosis developed P35 or P37 antibodies. Mice developed peak IgG titers to P35 and P37 within 30 days, followed by decline. Mice given both P35 and P37 antisera were protected from challenge with 10⁷ B. burgdorferi, and P35 and P37 antisera also afforded protection when administered 24 hr after spirochete challenge. The use of in vivo-expressed antigens such as P35 and P37 represents a new approach for Lyme disease serodiagnosis and for understanding the role of B. burgdorferi-specific immune responses in host immunity.

Introduction

Borrelia burgdorferi, the spirochetal agent of Lyme disease, undergoes dramatic alterations of its outer surface proteins during different stages of its life cycle in ixodes scapularis (also known as Ixodes dammini) ticks and in the mammalian host (Barthold et al., 1995; Fingerle et al., 1995; Schwan et al., 1995; de Silva et al., 1996). For example, outer surface protein (Osp) C is not expressed by spirochetes within unfed ticks; however, following introduction of a blood meal into the tick midgut, B. burgdorferi synthesize OspC (Schwan et al., 1995). Conversely, OspA is a prominent antigen on spirochetes within the midguts of flat ticks and is down-regulated or lost while spirochetes migrate from the midgut to the salivary gland in the feeding tick (de Silva et al., 1996). Once spirochetes have entered the mammalian host, they are no longer vulnerable to OspA-induced immunity (Barthold et al., 1995; de Silva et al., 1996). OspC and OspA serve as a paradigm for the significant changes that occur in different adaptive forms of the spirochete.

Other evidence for variation of B. burgdorferi gene expression within the vertebrate host also exists: eppa, pG, bbk2.10, and several ospE/F homologs appear to be selectively expressed in vivo (Champion et al., 1994; Wallich et al., 1995; Akins et al., 1996; Marconi et al., 1996; Montgomery et al., 1996; Stevenson et al., 1996). The humoral response to B. burgdorferi antigens expressed within the vertebrate host may also aid in the serologic diagnosis of Lyme disease. Enzyme-linked immunosorbent assay (ELISA) and immunoblots based on whole-cell lysates of B. burgdorferi may not detect antibodies directed toward proteins preferentially expressed in vivo because these antigens may not be present on cultured spirochetes (Magnarelli, 1989; Dresler et al., 1993; Johanson et al., 1996). The inability to detect antibodies directed toward in vivo-expressed antigens may be one reason why current serologic tests for Lyme disease remain difficult to interpret. In addition, the humoral response of B. burgdorferi–infected mice to in vivo-expressed proteins has powerful biologic effects, which may lead to the discovery of new vaccine strategies. Very small amounts of sera from actively infected mice (immune sera), when passively transferred to naive mice, confer protection against high-dose B. burgdorferi challenge. Remarkably, the protective activity of immune sera peaks at 30 days of infection, then declines throughout the course of persistent infection, unlike the progressive rise in antibody directed against antigens derived from cultured B. burgdorferi (Barthold and Bockenstedt, 1993). Immune sera have also been shown to induce arthritis resolution in actively infected severe combined immunodeficient (SCID) mice (Barthold et al., 1996). Thus, identification of in vivo-expressed antigens of B. burgdorferi offers the opportunity for the development of more accurate serodiagnostic antigens, preventive vaccines, and possibly therapeutic vaccines.

We recently developed a differential screening strategy to identify B. burgdorferi genes that may be selectively induced in vivo. In this approach, a B. burgdorferi genomic DNA expression library was probed with two sets of sera: immune sera from mice infected with B. burgdorferi and hyperimmune sera from mice immunized with killed spirochetes (Suk et al., 1995). Plaques reactive with immune, but not hyperimmune, sera reflect genes that are differentially expressed in vivo. One such identified gene, p21, was shown to be expressed in vivo using Northern blot and reverse transcriptase polymerase chain reaction (RT-PCR), and 14 additional phage plaques that correspond to five groups of genes and that may encode other in vivo-expressed proteins were isolated (Suk et al., 1995). In this report we describe the identification of two immunogenic proteins, P35 and P37, that are expressed early during B. burgdorferi infection of mice and that are recognized by serum from human Lyme disease patients. P35 and P37 are promising targets for diagnostic tests or vaccines for the prevention of Lyme disease.

Results

Identification of p35 and p37

Previous differential screening of a lambda ZAP II B. burgdorferi expression library with immune serum from mice infected with B. burgdorferi for 90 days and also with hyperimmune serum from mice immunized with heat-killed spirochetes revealed 14 phage clones that represented five B. burgdorferi genes (or members of gene families) that expressed antigens selectively reactive with immune sera (Suk et al., 1995). Two of these five groups of clones now also showed strong reactivity with antibodies in the immune sera of 30-day infected mice. The genes encoding these two immunogenic antigens were sequenced and designated p35 and p37. p35 and p37 had open reading frames of 927 and 996 nucleotides, respectively (Figures 1A and 1B). – 35 and 10 regions as well as ribosome binding sites were identified upstream of the respective open reading frames. The deduced amino acid sequence of P37 revealed a phenylalanine-X-Y-cysteine motif and a hydrophobic leader sequence, consistent with a lipoprotein. Pulsed-field gel electrophoresis demonstrated that both these genes are encoded on different plasmids: p35 and p37 are on plasmids with the mobility of a 42 and 16 kb linear DNA, respectively (Figure 1C).

p35 and p37 Are Expressed In Vivo

Because the differential immunologic screening suggested that these antigens are selectively synthesized in vivo, we performed further molecular studies. RNA from cultured B. burgdorferi was probed with p35 and p37 in Northern blot analyses. p35 and p37 RNA were not detected, while ospA RNA (control) was identified (Figure 2A). In addition, ospA, but not p35 or p37, could be identified in RNAse protection assay using RNA from cultured spirochetes (Figure 2C) and RT-PCR using RNA from cultured B. burgdorferi showed a band consistent with ospA, but not p35 or p37 (Figure 2B). To ensure that the B. burgdorferi used in the assays contained p35 or p37 DNA, Southern blot analyses were performed: as expected, DNA for ospA, p35, and p37 could be detected (Figure 2D).

We then used a variety of strategies to show that p35 and p37 were selectively expressed in vivo. Total RNA was harvested and subject to RT-PCR. In the first few days of infection, when spirochetes are localized to the site of inoculation, p35 and p37 mRNA could not be detected in the skin (at the place of spirochete injection), whereas flagellin mRNA (control) was readily amplified (Figures 3A and 3B). As expected, p35, p37, or flagellin mRNA could not be identified at distant skin sites or the spleen (Figure 3E) at these early time points because spirochetes had not yet disseminated from the inoculation site and could not be cultured from spleen or skin. At 7 and 14 days, when spirochetes had migrated from the site of inoculation, p35 and p37 mRNA became detectable at the initial site of infection (Figures 3C and 3D). In addition, p35 and p37 mRNA could be amplified by RT-PCR from distant skin and spleen that had been infected for 7 or 14 days (Figures 3F, 3G, and 3H).

Mice developed a strong humoral response to p35 and p37 following infection with 10^6 B. burgdorferi, further suggesting that these antigens are expressed in vivo (Figures 4A and 4B). Immunoglobulin (Ig) G to both P35 and P37 could be detected as early as 14 days after infection and peaked at 30 days. By 60–90 days following infection, these antibody responses had diminished and practically disappeared by 180 days. In contrast, IgG to B. burgdorferi whole-cell lysates continued to increase with persistent infection (Figure 4C). Since spirochetes are normally transmitted by tick bite, we measured the antibody response to P35 and P37 in mice infected with B. burgdorferi following tick bite. At 14 days following tick-borne infection, antibodies to P35 and P37 were detectable by immunoblot (Figure 4D).

Patients with Lyme Disease Develop P35 and P37 Antibodies

We assessed the human IgM and IgG responses to P35 and P37 using a reference panel of 43 sera from patients with well-documented Lyme disease that were carefully selected by the Centers for Disease Control (Fort Collins, Colorado). Twenty-seven of the individuals were culture-positive for B. burgdorferi, including 25 patients with erythema migrans (skin) and 2 individuals with meningitis (cerebrospinal fluid). Overall, 31 of the 43 (72%) patients had IgG or IgM to P35 and/or P37 by ELISA, including 24 of 33 patients with early-stage disease and 7 of 10 patients with late-stage disease (Table 1). Thirty-four of these 43 patients (79%) tested positive for Lyme disease in immunoblots using B. burgdorferi whole-cell lysates as the substrate: the 9 patients who tested negative in both assays uniformly had early disease that was effectively treated with antibiotics, and were culture-positive from biopsy specimens of the erythema migrans lesion. IgM to P35 or P37 and IgG to P35 and P37 were evident in 11, 8, 23, and 25 of these 43 individuals, respectively. As shown in Figure 5, pooled sera (1:100 dilution) from a group of 5 individuals with well-documented Lyme disease had strong IgG and IgM responses to glutathione transferase (GT)-P35 (IgG: optical density [OD] 1.43 ± 0.24; IgM: OD 0.72 ± 0.15) and GT-P37 (IgG: OD 1.13 ± 0.31; IgM: OD 0.39 ± 0.08) and little reactivity to GT (IgG: OD 0.12 ± 0.04; IgM: 0.17 ± 0.04). The mean OD values for the 43 patient sera were as follows: GT-P35 (IgG 0.46 ± 0.12 and IgM 0.38 ± 0.14), GT-P37 (IgG 0.48 ± 0.10 and IgM 0.36 ± 0.09), and GT-P37 (IgG 0.11 ± 0.04 and IgM 0.25 ± 0.14). Representative ELISA results using sera from selected patients are presented in Figure 5. Sera from 4 healthy individuals, 5 patients with a history of syphilis and a reactive VDRL, and 5 patients with systemic lupus erythematosus or rheumatoid arthritis did not have detectable antibodies to P35 and P37.

P35 and P37 Elicit Protective Immunity against Experimental B. burgdorferi Infection

Because P35 and P37 are synthesized in the early stages of mammalian infection, we sought to determine whether the immune response to P35 and/or P37 could induce protective immunity in mice (Table 2). Vaccination with both P35 and P37 was protective against a
B. burgdorferi P35 and P37 Immunity In Vivo

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Figure 1. Sequences of p35 and p37

The nucleotide and deduced amino acid sequences are depicted for p35 (A) and p37 (B). The putative ribosome binding site (RBS) and –10 and –35 promoter regions are underlined. Asterisks indicate the stop codon. (C) B. burgdorferi DNA separated by pulsed-field gel electrophoresis and probed with p37 (lane 1) and p35 (lane 2). These sequences are available from GenBank, Bankit identification number 85537.
Immunity

Figure 3. p35 and p37 Are Expressed in Murine Skin 7 Days after Experimental Infection

Ethidium bromide-stained gel of RT-PCR products amplified from B. burgdorferi RNA recovered from skin or spleen 1, 2, 3, 7, or 14 days after inoculation with 10^5 spirochetes. Skin samples were obtained from the site of inoculation at 1 (A), 2 (B), 7 (C), and 14 (D) days. Skin samples were obtained from distant locations at 3 (E) and 14 (F) days, and spleen at 7 (G) and 14 (H) days. p35, p37, and flagellin (fla) primers were used for amplification (with +) and without (−) RT. M, molecular mass markers.

Discussion

These studies characterize p35 and p37, B. burgdorferi genes that appear to be expressed in the early stages of mammalian infection. Because B. burgdorferi can alter its antigentic structure, it is important to understand the extent to which variation occurs and the role that the immune response to selectively synthesized proteins plays in the evolution of Lyme disease. For example, while OspA is an abundant antigen on cultured spirochetes and on B. burgdorferi within flat ticks, OspA is down-regulated when ticks engorge (de Silva et al., 1996). The extent to which ospA is expressed during mammalian infection needs to be explored further, but several studies suggest that OspA is not abundant during infection. When OspA-immune mice are challenged with host-adapted spirochetes, they develop disseminated infection and disease, suggesting that spirochetes are not vulnerable to OspA immunity once adapted to the host (Barthold et al., 1995). Moreover, OspA antibodies are ephemeral during early human Lyme disease, and ospA mRNA is difficult to detect in the initial period of murine infection (Schutzer et al., 1994; Kalish et al., 1995; Montgomery et al., 1996). Alternatively, OspA antibodies in the later stages of murine and human infection (particularly in patients with antibiotic-resistant chronic arthritis), as well as the identification of OspA-specific T cells in individuals with Lyme arthritis, imply that at some point during infection ospA is expressed (Yssel et al., 1991; Fikrig et al., 1992; Kalish et al., 1993). The degree to which and the reasons why B. burgdorferi surface protein synthesis changes during the spirochetal life cycle are not known.
P35 and P37 were first identified by differentially screening a B. burgdorferi expression library with immune sera from B. burgdorferi-infected mice and animals hyperimmunized with heat-killed spirochetes. This technique previously identified p21, an ospE homolog that is induced in vivo (Suk et al., 1995). To identify p21, the library was probed with immune sera from mice infected with B. burgdorferi for 180 days. In contrast, when p35 and p37 were found, the library was screened with sera obtained from mice infected for 30 and 90 days, in an effort to facilitate the identification of genes that are induced in the early stages of infection. The detection of P35 and P37 with immune sera from mice during early but not later stages of infection may explain why p35 and p37 were not uncovered in the initial screening strategy (using 180-day immune sera) that revealed p21. The selective induction of p35 and p37 in the mammalian host and the lack of in vitro expression were confirmed by the detection of p35 and p37 mRNA in B. burgdorferi-infected murine tissue but not in cultured spirochetes, and the presence of P35 and P37 antibodies in the serum of infected mice and patients with Lyme disease.

The kinetics of both P35 and P37 antibody responses during persistent B. burgdorferi infection of mice are striking, with peak antibody responses within 30 days of infection, followed by decline. This suggests that these antigens are priming an immune response only during the early stages of infection. It is notable that identical antibody kinetics occur when the passive protective activity of immune serum is titrated in persistently infected mice. In contrast, IgG to B. burgdorferi whole-cell lysates increases from 30 to 90 days, and antibodies to an antigen that is likely to be constitutively expressed (flagellin) continue to be present in high titer. The parallel kinetics of P35 and P37 antibody with protective antibody kinetics suggests that P35 and P37 are among the antigens likely to be responsible for the protective activity in sera of actively infected mice.

Since p35 and p37 expression occurs early in infection, we sought to determine the role of P35 and P37 antibodies in immunity. Immune sera from B. burgdorferi-infected mice have the capacity to both protect mice from infection, eliminate infection up to 4 days after inoculation, and induce arthritis resolution in actively infected mice, whereas hyperimmune sera to heat-killed
Table 1. P35 and P37 ELISA Analysis of Sera from Patients with Lyme Disease

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B. burgdorferi-whole-cell lysate based immunoblots (IgM and IgG) were assessed by the Centers for Disease Control according to the banding criteria established by Dressler et al. (1993).

spirochetes is only protective but does not influence infection or disease after inoculation (Barthold et al., 1996). This unique capacity of immune serum to clear B. burgdorferi during early infection, suggests the response to in vivo induced antigens is responsible for this effect. Indeed, antibodies to OspA (with profound protective capabilities in vaccine studies) are abundant in the sera of mice hyperimmunized with heat-killed spirochetes but cannot eliminate the pathogen once infection is established (Fikrig et al., 1993). Sera from B. burgdorferi-infected mice recognized flagellin, P39, and OspC on immunoblot, suggesting that antibodies to these proteins may elicit the protective, postinfectious, or disease-modulating effect of immune serum. However, when antisera against these specific proteins were administered to B. burgdorferi-infected mice, no effects were noted (Barthold et al., 1996). Our current studies show that P35 and P37 antisera have the capacity to protect mice from infection, when administered both prior to and 24 hr after B. burgdorferi challenge. Therefore, P35 and P37 antibodies provides a basis for understanding the protective capacity of sera from B. burgdorferi-infected mice.

The data also indicate that p35 and p37 are expressed several days after the spirochetes are introduced into the skin. p35 and p37 mRNA could not be detected in biopsy specimens from the site of experimental inoculation for up to 48 hr but could be identified in skin and tissue specimens at 7 and 14 days. During experimental murine Lyme borreliosis, spirochetes disseminate from the initial skin lesion at 3-5 days and then spread hematogenously to visceral organs and other cutaneous sites (Barthold et al., 1991). p35 and p37 expression temporally coincides with the spread of B. burgdorferi, and this suggests that p35 and p37 may play a role in spirochete migration or tissue invasion.

The prominent humoral response to P35 and P37 in the early stages of murine and human Lyme borreliosis also suggests that P35 and P37 antibodies may be useful markers of infection. Current serologic assays, including ELISA and immunoblot, that detect exposure to B. burgdorferi use whole-cell lysates of cultured spirochete as the substrate and therefore cannot detect antibody responses to proteins expressed in vivo (Stanek et al., 1987; Magnarelli, 1989; Dressler et al., 1993). This is likely to result in an incomplete assessment of the immune response to B. burgdorferi. Studies analyzing the temporal development of the human and murine response to B. burgdorferi use whole-cell lysate-based systems have indicated that, in general, antibodies to flagellin, P39, and OspC appear in the early stages of disease and that antibodies to numerous spirochetal antigens develop as the infection continues (Dressler et al., 1993; Barthold et al., 1995; Barthold et al., 1996; Magnarelli et al., 1996). While antibodies to these proteins certainly are elicited during infection (suggesting that many antigens are expressed both in vitro and in vivo), the response to in vivo induced gene products is not discernible using standard assays. Our data suggest that the evolution of the humoral response is more complex that previously considered. Indeed, it is likely that several genes are induced or inhibited at various stages of infection and that current detection methods are unable to identify antibodies to these antigens because they lack the “in vivo” antigen. This paradigm may extend beyond Lyme disease and apply to other microbes that result in persistent infection.

Experimental Procedures

B. burgdorferi

B. burgdorferi isolate N40 (cloned N40) with proven infectivity and pathogenicity in mice was used throughout the study (Barthold et al., 1995). Spirochetes were grown in Barbour-Stoenner-Kelly (BSK) II medium at 35°C for use in all the experiments (Barbour, 1984).
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Figure 5. ELISA ( IgM and IgG) Using Recombinant GT-P35 or GT-P37 as the Substrate and Sera from Representative Patients with Lyme Disease

The serologic responses of 8 individuals, 5 with erythema migrans (the second and fourth patients were culture-positive), 3 with arthritis, and 1 with meningitis, are shown. Positive control sera (pos. control) are pooled from 5 patients with well-documented Lyme disease, from our clinic at Yale, with IgG or IgM antibodies to B. burgdorferi whole-cell lysates. Negative control sera are pooled from 3 healthy individuals.

Mice

Three-week-old female C3H/HeN Crr (C3H) mice were obtained from the Frederick Cancer Research Center (Frederick, MD). Mice were housed in filter-frame cages and euthanized with carbon dioxide.

Antibodies

Sera were obtained from mice infected (immune sera) or hyperimmunized (hyperimmune sera) with B. burgdorferi. For infection, mice were given with an intradermal inoculation of 10^6 B. burgdorferi N40. Sera were obtained from the animals at 7, 14, 28, 42, 60, 90, 180, and 270 days. All the mice had verified infection based on culture of spleen, urinary bladder, and/or skin. For hyperimmunization, groups of 5 mice were injected with 10^6 heat-killed spirochetes in complete Freund’s adjuvant (CFA) and given booster injections at 2 and 4 weeks with the same number of B. burgdorferi in incomplete Freund’s adjuvant (IFA). Mice hyperimmunized with heat-killed B. burgdorferi were culture-negative at the time of sacrifice. P35 or P37 antisera were prepared by hyperimmunizing a rabbit with 50 μg of either GT-P35 (recombinant P35) prepared as a glutathione transferase fusion protein) or GT-P37 (recombinant P37 prepared as a GT fusion protein) in CFA and boosting the animal twice over a period of 1 month; in each case the P35 or P37 antibodies were present at a titer of 1: 25,000 by immunoblot. GT antisera (control) were prepared in an identical fashion.

Library Screening

A lambda ZAP II genomic expression library of B. burgdorferi N40 was used (Lam et al., 1994). The phage plaques were probed with immune sera from 30 day-infected mice and hyperimmune sera from B. burgdorferi-hyperimmunized mice. Sera (1: 100 dilution in phosphate-buffered saline [PBS]) were used as the primary antibody. Secondary antibodies were goat anti-mouse immunoglobulin conjugated to alkaline phosphatase and were detected following incubation with nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl-phosphate (BCIP). Plaques that reacted uniquely with immune sera but not hyperimmune sera were subjected to secondary and tertiary screening. pBluescript plasmids that contained genes encoding reactive antigens were excised using the R408 helper phage (Lam et al., 1994). The nucleotide sequences of the genes encoded on the plasmid inserts were then determined using the Circumvent Thermal Cycle Dideoxy DNA sequencing kit (New England Biolabs) and designated p35 and p37 respectively, based on the estimated size of the gene products.

Pulsed-Field Gel Electrophoresis

Pulsed-field gel electrophoresis was performed with B. burgdorferi DNA (Lam et al., 1994). DNA plugs containing approximately 10^8 B. burgdorferi N40 were loaded onto a 0.8% agarose gel and run in Tris-borate-EDTA buffer (0.025 M Tris, 0.5 mM EDTA, 0.025 M boric acid), using the CHEF-DR II system (Bio-Rad Laboratories, Richmond, CA). The gel was run at 14°C for 18 hr at 198 V, with ramped pulse times from 1 to 30 s. Southern blotting was carried out with p35 and p37 probes labeled with (α-32P) dCTP, using the Prime It random primer kit (Stratagene, La Jolla, CA).

Northern Blot Analysis

Total RNA was isolated from cultured B. burgdorferi by acid guanidinium thiocyanate phenol-chloroform extraction. Ten micrograms of the recovered RNA was electrophoresed in a 1% formaldehyde-agarose gel and blotted onto a Hybond-N membrane. The blotted membrane was prehybridized in 50% formamide for 1 hr at 42°C, and then hybridized, using the same conditions, with probes for p35, p37, or ospA overnight. Random-primed 32P-labeled DNA inserts encoding p35 (920 bp), p37 (960 bp) and ospA (800 bp) were used as probes. The membranes were washed in 0.1 x SSC and 0.1% SDS at 55°C prior to exposure and development of film.

RNAse Protection Assays

The extracted RNA from cultured B. burgdorferi was hybridized with a 32P-labeled cRNA probe made from subcloned DNA containing selected B. burgdorferi genes, p35 (Accl, 190 bp), p37 (XmnI, 390 bp) and ospA (Psfl, 180 bp). The DNAs that spanned regions of p37 (nucleotides 606-996), p35 (nucleotides 737-927), and ospA (nucleotides 642-822) were subcloned into pBluescript. The antisense and sense 32P-labeled cRNA probes were transcribed using T7 and T3 polymerase. Template DNA was removed using RNAse-free DNase. The RPA II ribonuclease protection assay kit (Ambion, Austin, TX) was utilized for hybridization, RNAse digestion and electrophoretic separation. The gel was exposed to film for 24 hr. Sense probes produced no hybridization.

PCR

Conditions for PCR amplification of B. burgdorferi DNA were 30 cycles with a denaturing, annealing and extension temperature of 94°C for 1 min, 55°C for 1 min, and 72°C for 2 min. 5′ and 3′ primers for PCR were 5′-GGTGAAGTTGCTATGGG-3′ and 5′-CTTA GCAAATGGTGCAACCGGTG-3′ for p35; 5′-GACAA CACGAAAGTGG-3′ GTGCGAC-3′ and 5′-TAGCATCGGAATGCGTGC-3′ for p37; 5′-TCTGAGCATGCTACTGGCTACACA-3′ and 5′-CCCTCTAATTTGTGGTGGCA ATTG-3′ for ospA; and 5′-CATACTGATTACGTACCC-3′ and 5′-TCTATTGTTGAAAGC-3′ for flagellin. For RT-PCR, total RNA was isolated from the spleens, or the skin (5 mm section) of mice infected with...
with B. burgdorferi for 14 days, or from spirochetes cultured in BSK II medium, by acid guanidinium thiocyanate-phenol-chloroform extraction (Suk et al., 1995). Pooled RNA was then treated with DNase and RNA inhibitor (Promega, Madison, WI) for 3 hr at 37°C. Two micrograms of pooled RNA was reverse transcribed by RTh DNA polymerase (Perkin Elmer, Branchburg, NJ). "Downstream" primers for either p35, p37, or ospA were then added. The reactions were carried out at 70°C for 15 min. "Upstream" primers and MgCl₂ solution were then added as suggested by the manufacturer. PCR was carried out for 30 cycles of 94°C for 1 min, 53°C for 1 min, and 72°C for 2 min.

### Protein Purification

Recombinant P35 and P37 were expressed as fusion proteins with GT. The p35 and p37 genes were cloned into pMX, a modified pGEX-2T vector (Sears et al., 1991). To facilitate subcloning, the p35 and p37 genes were amplified using 5’ primers with an additional XhoI (for p35) and BamHI (for p37) restriction enzyme digestion site and a 3’ primer with a supplementary HindIII (for p35) or XhoI (for p37) site. Plasmid DNA obtained from initial phage colonies was used as the template for p35 and p37. Amplified gene products were digested with either XhoI and BamHI or HindIII and XhoI and cloned into the corresponding sites in the pMX plasmid. The ligation mixture was then used to transform Escherichia coli DH5α, and colonies containing the recombinant plasmid were isolated on Luria broth supplemented with ampicillin.

Recombinant fusion protein production was induced by growing the bacteria to logarithmic phase and adding 1 mM isopropyl-1-thio-β-D-galactoside (IPTG) for 3 hr. The E. coli were then placed in PBS with 1% Triton and subjected to sonication. The cell supernatant and pellet were separated by centrifugation at 10000 × g for 8 min. Recombinant fusion proteins present in the supernatant were then purified by passage over a glutathione column (Igirig et al., 1990). The fusion proteins, GT-P35 or GT-P37, were eluted from the column using a solution containing excess glutathione and quantified. Recombinant GT (control antigen) was prepared in an identical fashion. Purified p35 and p37, without the GT fusion partner, were also obtained, for the pMX vector contains a thrombin cleavage site immediately following the GT protein. After the supernatant containing the recombinant p35 or p37 fusion protein was passed onto the glutathione column, a solution containing thrombin was added to the column to cleave the recombinant proteins from the glutathione transferase.

### ELISA

For ELISA, duplicate sets of 96-well microtiter plates were coated with antigen (200 μg [1 μg/ml, 200 μl/well] of recombinant GT, GT-P35, or GT-P37) and incubated overnight at 4°C. Plates were then washed three times with 0.05% PBS Tween (PBST). Triplicate samples of patient or murine immune sera (200 μl/well, diluted 1:100) were then applied to the coated plates and incubated for 1 hr at room temperature. Plates were then washed three times with PBST. Goat anti-human or goat anti-mouse IgM and IgG, each diluted 1:2000 and linked to alkaline phosphatase, were then added to each well. Plates were incubated at room temperature for 1 hr and washed three times with PBST, and then 200 μl of freshly prepared p-nitrophenol phosphate, (1 mg/ml in glycine buffer, pH 10.5) was added to each well and monitored at 405 nm. The reaction was stopped with 3 M NaOH.

### Immunobots

Recombinant GT-P35 or GT-P37 were resolved in 12% minigels by SDS PAGE and transferred to nitrocellulose membranes. One microgram of recombinant antigen was loaded on each lane. The nitrocellulose strips containing recombinant protein were then probed with antisera (diluted 1:100). The secondary antibody was alkaline phosphatase conjugated goat anti-mouse or anti-human immunoglobulin. Color development was performed using NBT and BCIP.

### Immunization Studies

Groups of 5 mice were hyperimmunized subcutaneously with 10 μg of recombinant GT-P35 and/or GT-P37 in CFA and boosted at 14 and 28 days with the same amount of antigen in IFA, according to published protocols (Igirig et al., 1990). Control mice were hyperimmunized with recombinant GT in an identical fashion. Fourteen days following the last boost, sera from the hyperimmunized animals was used to probe recombinant P35 or P37 (prepared without the GT fusion partner) to determine the presence and titer of specific antibody. Hyperimmunized mice were challenged 14 days after the last boost with intradermal inoculations of 10° or 10⁴ spirochetes. In passive immunization studies, mice were administered 0.2 ml of GT-P35 and/or GT-P37 hyperimmune sera. Control mice were given GT hyperimmune sera. Passively immunized mice were challenged with 10° or 10⁴ B. burgdorferi either 24 hr prior to or 24 hr after passive immunization. In all the studies, mice were killed 14 days after challenge.

At necropsy, blood, spleen, urinary bladder and skin punches were obtained, for the pMX vector contains a thrombin cleavage site immediately following the GT protein. After the supernatant containing the recombinant p35 or p37 fusion protein was passed onto the glutathione column, a solution containing thrombin was added to the column to cleave the recombinant proteins from the glutathione transferase.

### Table 2. Lyme Borreliosis in C3H Mice Immunized with P35 or P37

<table>
<thead>
<tr>
<th>Immunogen</th>
<th>Challenge (no. of spirochetes)</th>
<th>Arthritis</th>
<th>Carditis</th>
<th>Culture-positive</th>
<th>Infection</th>
</tr>
</thead>
<tbody>
<tr>
<td>Active</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P35</td>
<td>10⁴</td>
<td>5/9</td>
<td>5/9</td>
<td>5/9</td>
<td>5/9</td>
</tr>
<tr>
<td>P37</td>
<td>10⁴</td>
<td>6/9</td>
<td>8/9</td>
<td>8/9</td>
<td>8/9</td>
</tr>
<tr>
<td>P35 and P37</td>
<td>2/10</td>
<td>1/10</td>
<td>2/10</td>
<td>2/10</td>
<td>2/10</td>
</tr>
<tr>
<td>GT (control)</td>
<td>10⁴</td>
<td>7/12</td>
<td>9/12</td>
<td>11/12</td>
<td>11/12</td>
</tr>
<tr>
<td>Passive</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P35 antiserum</td>
<td>10⁴</td>
<td>4/5</td>
<td>3/5</td>
<td>4/5</td>
<td>4/5</td>
</tr>
<tr>
<td>P37 antiserum</td>
<td>10⁴</td>
<td>3/5</td>
<td>3/5</td>
<td>3/5</td>
<td>3/5</td>
</tr>
<tr>
<td>P35 and P37 antiserum</td>
<td>10⁴</td>
<td>3/5</td>
<td>3/5</td>
<td>3/5</td>
<td>4/5</td>
</tr>
<tr>
<td>GT (control) antiserum</td>
<td>10⁴</td>
<td>0/7</td>
<td>0/7</td>
<td>0/7</td>
<td>0/7</td>
</tr>
</tbody>
</table>

In the active immunization studies, P35- and/or P37- or GT (control)-vaccinated mice were challenged with inocula of 10° or 10⁴ B. burgdorferi 14 days after the final boost and then killed at 14 days. In the passive immunization studies, mice were administered rabbit P35- and/or P37 hyperimmune sera or anti-GT hyperimmune serum (control). The antisera were given to the animals 1 day prior to or 24 hr after (24 hr post) challenge with B. burgdorferi. Blood, urinary bladder, spleen, and skin punches were cultured in BSK II medium for 2 weeks and examined by dark-field microscopy for spirochetes. The tibiotarsi and heart were examined microscopically for inflammation. Mice from which B. burgdorferi were isolated from at least one tissue specimen and/or had evidence of disease were considered to be infected.
from the animals were collected aseptically, cultured in BSK II medium, and examined by dark-field microscopy for spirochetes. The joints and the hearts were formalin fixed, paraffin embedded, sectioned, and examined microscopically for inflammation. Tibiotarsal arthritis was graded by blinded analysis on a scale from 1 (mild) to 3 (severe) as described (Fikrig et al., 1995).

Acknowledgments

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References


