Molecular Microbiology (1999) **31**(1), 281–290

Borrelia burgdorferi erpT expression in the arthropod vector and murine host

Erol Fikrig,[1] Manchuan Chen,[2] Stephen W. Barthold,[3] Juan Anguita,[1] Wen Feng,[2] Sam R. Telford III[4] and Richard A. Flavell[2,5]*

[1]Section of Rheumatology, Department of Internal Medicine, and [2]Section of Immunobiology, Yale University School of Medicine, New Haven, CT 06520, USA.
[3]Center for Comparative Medicine, Schools of Medicine and Veterinary Medicine, University of California, Davis, CA 95616, USA.
[4]Department of Tropical Public Health, Harvard University School of Public Health, Boston, MA 02115, USA.
[5]Howard Hughes Medical Institute, Yale University School of Medicine, New Haven, CT 06520, USA.

Summary

The expression of a *Borrelia burgdorferi* gene, *erpT*, was investigated throughout the spirochaete life cycle in the arthropod vector and the murine host. Three phage clones from a *B. burgdorferi* DNA expression library synthesized a 30 kDa antigen that was recognized by antibodies in the sera of *B. burgdorferi*-infected mice but not mice hyperimmunized with *B. burgdorferi* lysates. Differential antibody binding suggested that this protein was preferentially expressed *in vivo*. This antigen was designated ErpT, based upon 99.6% homology with the BBF01 sequence in the *B. burgdorferi* genome. ErpT was not detected on spirochaetes cultured in BSK II medium by indirect immunofluorescence or in *B. burgdorferi* lysates by immunoblotting, implying that ErpT is not readily produced *in vitro*. *erpT* mRNA was not discernible by Northern blot but was identified by RNA polymerase chain reaction *in vitro*, indicating that *erpT* is expressed at low levels by cultured spirochaetes. *erpT* expression was then investigated in the vector and mice because *B. burgdorferi* do not normally reside in culture medium. RNA polymerase chain reaction and immunofluorescence studies demonstrated that *erpT* was expressed by a small minority of *B. burgdorferi* (11/500, 2.2%) within unfed ticks and then repressed during engorgement. *erpT* mRNA or ErpT antibodies were first detected in *B. burgdorferi*-infected mice at 4 weeks, suggesting that *erpT* was not expressed in the early stages of murine infection. Then, during persistent infection, RNA polymerase chain reaction showed that *erpT* was expressed by *B. burgdorferi* within the joints, heart and spleen, but not by spirochaetes in the skin. Immunization of mice with ErpT was antigenic but was not protective. These studies demonstrate that *B. burgdorferi erpT* is differentially expressed throughout the *B. burgdorferi* life cycle, in both the vector and the mammalian host, and is primarily expressed in extracutaneous sites during murine infection.

Received 2 May, 1998; revised 2 September, 1998; accepted 17 September, 1998. *For correspondence. E-mail richard.flavell@qm.yale.edu; Tel. (203) 737 2216; Fax (203) 785 7561.

Introduction

Borrelia burgdorferi selectively expresses genes in ticks and the mammalian host, most probably in response to the vastly different environments that the bacterium must adapt to in these situations. For example, outer surface protein (Osp) A is present on spirochaetes within unfed but not within engorged ticks (de Silva *et al*., 1996; Coleman *et al*., 1997), and OspC is preferentially synthesized in feeding ticks (Schwan *et al*., 1995). Several genes, including *eppa*, *p35*, *p37*, *vls* and *ospE/F* paralogues (*ospE*-related proteins, *erps*) have been shown to be expressed by *B. burgdorferi* during infection of the mammalian host (Champion *et al*., 1994; Das *et al*., 1997; Fikrig *et al*., 1997; Zhang *et al*., 1997; Stevenson *et al*., 1998). *Borrelia burgdorferi* cultured in Barbour–Stoenner–Kelly (BSK) II medium *in vitro* represent an artificial, but nevertheless important, environment in which *B. burgdorferi* gene expression can also be investigated. We developed a differential immunological screening strategy to identify *B. burgdorferi* genes selectively expressed during infection (Suk *et al*., 1995). In this technique a *B. burgdorferi* genomic DNA expression library was probed with sera from mice infected with *B. burgdorferi* (immune sera) and with sera from mice immunized with killed *B. burgdorferi* whole-cell lysates (hyperimmune sera) (Suk *et al*., 1995). Clones that reacted with immune sera, but not hyperimmune sera, reflected genes that are likely to be selectively expressed during infection (Suk *et al*., 1995). Using this approach, we initially discovered at least five genes (or gene families) that appeared to be expressed during mammalian infection (Suk *et al*., 1995). Immunological

and molecular studies have characterized three of these five genes: *p21*, *p35* and *p37* (Das *et al*., 1997; Fikrig *et al*., 1997). *p21* was not expressed by *B. burgdorferi* within ticks and only induced after 21–28 days of infection in mice (Das *et al*., 1997). *p35* and *p37*, which are unrelated genes, were expressed at an earlier time point (day 7) during infection of mice, when the spirochaetes disseminate from the site of skin entry to the bloodstream and distant organs (Fikrig *et al*., 1997).

Selective gene expression by *B. burgdorferi* could possibly aid in spirochaete infectivity, tissue tropism, metabolism or evasion of host defences. Downregulation of the highly immunogenic OspA by spirochaetes in the engorging tick renders them resistant to the borreliacidal effects of OspA antibodies (de Silva *et al*., 1996). *Borrelia burgdorferi* do not, however, repress *ospA* in response to the presence of OspA antibodies: downregulation of OspA occurs after the incoming bloodmeal from naive hosts (Coleman *et al*., 1997; de Silva *et al*., 1996). On the other hand, larval ticks cannot acquire *B. burgdorferi* when the host is passively administered OspA antibodies (de Silva *et al*., 1997). Effective completion of the *B. burgdorferi* life cycle from tick to mammal to tick therefore requires that spirochaetes do not readily express *ospA* – and thereby elicit an OspA response – in the mammal. This provides a partial explanation for the downregulation of OspA as *B. burgdorferi* migrates from the vector to the host. Antibodies to additional gene products that are selectively synthesized at different points in the *B. burgdorferi* life cycle, such as OspE/F paralogues (Erps), OspC, P35 and P37, also have borreliacidal properties and can partially protect a host from *B. burgdorferi* challenge, or may affect disease and spirochaete clearance during an ongoing infection (Nguyen *et al*., 1994; Schwan *et al*., 1995; Stevenson *et al*., 1996; Barthold *et al*., 1997; Fikrig *et al*., 1997; Zhong *et al*., 1997). Differential expression of these genes, or the selection of antigenic variants, could contribute to spirochaete persistence. However, it has not been shown that expression of these genes is related to host antibodies. Selectively expressed genes may also aid in pathogenesis. The binding of host plasminogen to *B. burgdorferi* in the tick and the mammalian host enhances infectivity, and OspA is one of at least several *B. burgdorferi* plasminogen binding proteins (Fuchs *et al*., 1994; Coleman *et al*., 1997). Moreover, *p35* and *p37* are expressed as the spirochaetes migrate from the skin to distant organs, suggesting a role in dissemination (Fikrig *et al*., 1997).

We have now identified a plasmid-encoded *B. burgdorferi* gene, designated *erpT*, that is expressed by some spirochaetes within unfed ticks, then repressed within engorged ticks, and then expressed again during persistent murine infection. *ErpT* expression in mice occurs primarily in extracutaneous tissues. The characterization of *erpT* expression by *B. burgdorferi* within ticks and in the mammalian host contributes to our understanding of selective gene expression during different stages of the lifecycle of spirochaetes.

Results

Identification of erpT

A *B. burgdorferi* genomic DNA expression library was differentially probed with sera from mice infected with *B. burgdorferi* (immune sera) and mice immunized with *B. burgdorferi* lysates (hyperimmune sera). Fourteen phage clones synthesized antigens that selectively reacted with immune sera and represented at least five genes (Suk *et al*., 1995). Three of these five genes, *p21*, *p35* and *p37* have previously been shown to be expressed by *B. burgdorferi* in the mammalian host, but not by spirochaetes cultured *in vitro* (Suk *et al*., 1995; Das *et al*., 1997; Fikrig *et al*., 1997). These genes were present on 8 of these 14 phage clones. We now characterize an additional gene that was identified using this screening strategy. Three of the remaining six phage clones contained DNA encoding an 825 nucleotide open reading frame, designated *erpT* because of 99.6% homology with the BBF01 sequence from the *B. burgdorferi* B31 genome (Fraser *et al*., 1997). *Borrelia burgdorferi* N40 *erpT* is 170 nucleotides shorter than the *B. burgdorferi* B31 homologue, and the sequence has been submitted to GenBank (accession number AF043236). Pulsed-field gel electrophoresis showed that *erpT* was on a plasmid with the mobility of 28 kb linear DNA in *B. burgdorferi* N40, consistent with data from the *B. burgdorferi* B31 genome indicating that *erpT* is present on the lp-28-1 linear plasmid (Fraser *et al*., 1997). Although *erpT* has 54% identity with *erpD*, binding of an *erpT* probe to plasmids that encode *ospE/F* paralogues (Lam *et al*., 1994; Akins *et al*., 1996; Stevenson *et al*., 1996; Das *et al*., 1997; Stevenson *et al*., 1998) was not noted on pulsed-field electrophoresis.

erpT *is expressed by* B. burgdorferi *during murine infection*

Studies investigated whether *erpT* was expressed by *B. burgdorferi* cultured *in vitro* and during mammalian infection. *erpT* mRNA could not be detected by Northern blot in *B. burgdorferi* cultured in BSK II medium (Fig. 1A). Northern blots were exposed for 18 h (Fig. 1A) and *erpT* mRNA could not be detected when the blots were exposed for up to 72 h. *Borrelia burgdorferi* mRNAs that are less abundant than *ospA*, such as *flaB*, *ospE*, *ospF* and *ospD*, could also be detected by northern blot (not shown). In addition, ErpT could not be identified using ErpT antisera in *B. burgdorferi* extracts by immunoblotting (Fig. 1B, lanes 1 and 2) or on cultivated spirochaetes by immunofluorescence (IFA) (Fig. 1C). As expected, ErpT

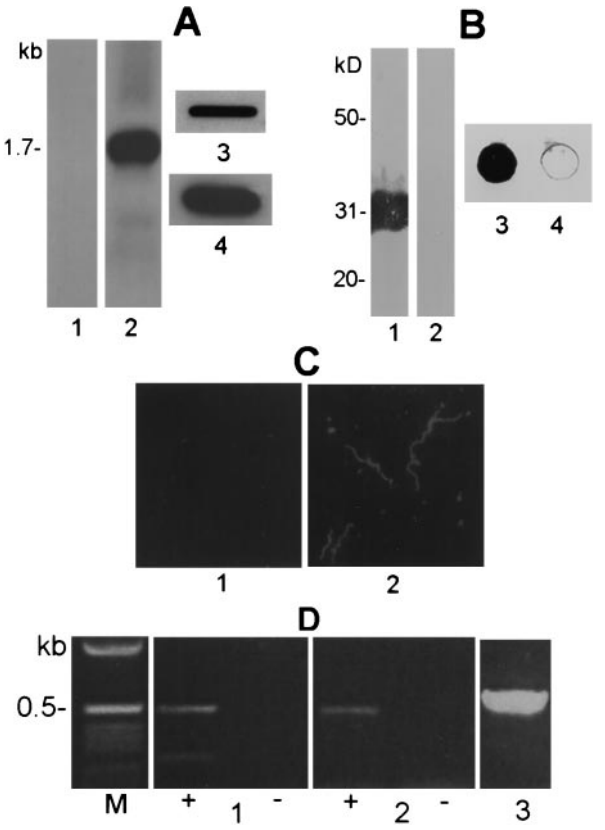

Fig. 1. *Borrelia burgdorferi erpT* is not readily expressed *in vitro*.
A. Northern blot of *B. burgdorferi* mRNA probed with *erpT* (lane 1) or *ospA* (lane 2), Southern blot of *B. burgdorferi* DNA probed with *erpT* (lane 3) or *ospA* (lane 4).
B. Immunoblot of *B. burgdorferi* lysates probed with OspA antisera (lane 1) or ErpT antisera (lane 2). Dot-blot of recombinant ErpT probed with ErpT antisera (lane 3) or GT antisera (lane 4).
C. Immunofluorescence of laboratory-cultured *B. burgdorferi* probed with ErpT antisera (1) or *B. burgdorferi* antisera (2).
D. RNA PCR detection of *B. burgdorferi* mRNA using *erpT* (1) or *ospA* primers (2). With (+) or without (−) reverse transcriptase. PCR of *B. burgdorferi* DNA using *erpT* primers (3).

antisera readily recognized recombinant ErpT (Fig. 1B, lanes 3 and 4). *erpT* mRNA could be found, however, in cultured spirochaete, albeit at a low level, by RNA polymerase chain reaction (PCR) (Fig. 1D).

erpT expression by *B. burgdorferi* during murine infection was then evaluated by RNA PCR. Mice were infected with an intradermal inoculation of 10^4 *B. burgdorferi*. Animals were killed at 14, 30, 60 or 90 days. At 14–30 days, arthritis and carditis are most severe, whereas disease resolves at 60–90 days (Barthold *et al*., 1991). At 14 days, *erpT* mRNA was not detected in murine tissues. At 30 days, *erpT* mRNA was found in the skin, spleen, joints, heart and bladder. At 60 and 90 days, *erpT* mRNA was evident in all tissues except the skin. In contrast, *flaB* mRNA (control) was detected at all time points and in all locations. *ospA* mRNA was initially apparent after challenge (day 14 in the skin and spleen), and then not detected during persistent infection. Table 1 summarizes the RNA PCR studies. In all PCR studies, reactions without the addition of reverse transcriptase (RT) did not yield a signal, indicating that DNA contamination was not evident. Overall, these data suggested that *erpT* was not expressed by *B. burgdorferi* in the first weeks of murine infection but was induced at later time points and primarily in extracutaneous sites. To simulate the natural mode of transmission, *erpT* expression was also examined during tick-borne infection. At 14 days, *erpT* mRNA was not detected in mice infected with *B. burgdorferi* by tick bite. At 30 days, *erpT* mRNA was evident in all the tissues examined (joints, heart, spleen and bladder) except the skin. This is generally consistent with the studies using an intradermal spirochaete challenge, suggesting that *erpT* is not predominantly expressed in the skin.

To determine whether the ability to detect *B. burgdorferi*-specific mRNAs was influenced by the number of organisms in specific tissues, serial dilution PCR studies were performed to quantify *B. burgdorferi* at several of the examined sites, using previously described procedures (Anguita *et al*., 1996). Samples were obtained from each of the tissue specimens and used for DNA extraction. An equal amount of the extracted DNA (≈1 mg) was used as the initial template for serial dilution PCR. At 14 days after infection, 1 mg of DNA from a skin tissue specimen contained a mean of 480 000 ± 30 000 standard error (SE) *B. burgdorferi*. At this time point 1 mg of DNA from heart tissue contained a mean of 800 000 ± 280 000 SE spirochaetes, and 1 mg of DNA from a tibiotarsal joint contains a mean of 340 000 ± 90 000 SE spirochaetes.

Table 1. Expression of *erpT*, *flaB* and *ospA* during murine Lyme borreliosis.

	B. burgdorferi mRNA in specific murine tissues														
	Skin			Spleen			Heart			Joints			Bladder		
Day of infection	*erpT*	*flaB*	*ospA*	*erpT*	*flaB*	*ospA*	*erpT*	*flaB*	*ospA*	*erpT*	*flaB*	*ospA*	*erpT*	*flaB*	*ospA*
14	−	+	+	−	+	+	−	+	−	−	+	−	−	+	−
30	+	+	−	+	+	−	+	+	−	+	+	−	+	+	−
60	−	+	−	+	+	−	+	+	−	+	+	−	+	+	−
90	−	+	−	+	+	−	+	+	−	+	+	−	+	+	−

mRNA was isolated from the skin, spleen, heart, tibiotarsal joints and bladder at selected time points after challenge with *B. burgdorferi* and subjected to RNA PCR using *erpT*, *flaB* and *ospA* primers.

At 30 days after infection, the spirochaete burden in these tissues decreased to 50 000 + 20 000 SE and 60 000 + 30 000 SE organisms per heart and joint specimen, respectively, and 75 000 + 20 000 SE *B. burgdorferi* per skin specimen. *Borrelia burgdorferi* numbers in the skin, joints and heart at 60 days were similar (no statistical differences) from 30 days. Therefore, the inability to detect *erpT* mRNA in the tissues, particularly at day 14 and in the skin at later time points, was not due to the relative lack of spirochaetes in these tissues.

Quantitative RNA PCR was also used to estimate the relative abundance of *erpT* mRNA in the skin, hearts and joints at 30 and 60 days. *erpT* mRNA (19.0 ag) (95% confidence interval, 16.6–21.4) could be detected in the skin at 30 days, whereas 6.6 ag (95% confidence interval, 5.4–7.8) and 5.9 ag (95% confidence interval, 3.9–7.9) of *erp*T mRNA was evident in the heart and joints respectively. At 60 days, *erpT* mRNA levels decreased to 2.9 ag (95% confidence interval, 2.1–3.7) and 1.5 ag (95% confidence interval, 0.9–2.1) in the heart and joints, respectively, and could not be detected in the skin. These data suggest that e*rpT* mRNA levels in the skin at 30 days are similar to (if not increased) when compared with the other tissues at this interval.

erpT *expression by* B. burgdorferi *within ticks*

Borrelia burgdorferi undergoes differentially gene expression within ticks during engorgement. Therefore, *erpT* expression was examined in both unfed and engorged ticks (Fig. 2). *erpT* mRNA was detected by RNA PCR within unfed ticks but not engorged *Ixodes scapularis* (Fig. 2A). *flaB* mRNA (control) was evident in both flat and engorged ticks. ErpT was discernible on some (11/500, 2.2%) of the *B. burgdorferi* within flat ticks by IFA (Fig. 2B), and was not apparent on spirochaetes within engorged ticks (0/600, *t*-test $P < 0/001$). The panel demonstrating the spirochaetes in unfed ticks that synthesize ErpT (Fig. 2B) represents a rare occurrence. Most of the high-power fields examined do not contain ErpT-synthesizing spirochaetes, and contain several (1–5) spirochaetes that bind *B. burgdorferi* antisera. This panel in Fig. 2B contains a single long spirochaete or two *B. burgdorferi* that are tethered together; for the purposes of counting, this was considered as one spirochaete. *Borrelia burgdorferi* could be readily visualized in unfed or fed ticks using a *B. burgdorferi* antisera. These data suggest that *erpT* is expressed by some spirochaetes in flat ticks and is not expressed by *B. burgdorferi* in engorged ticks.

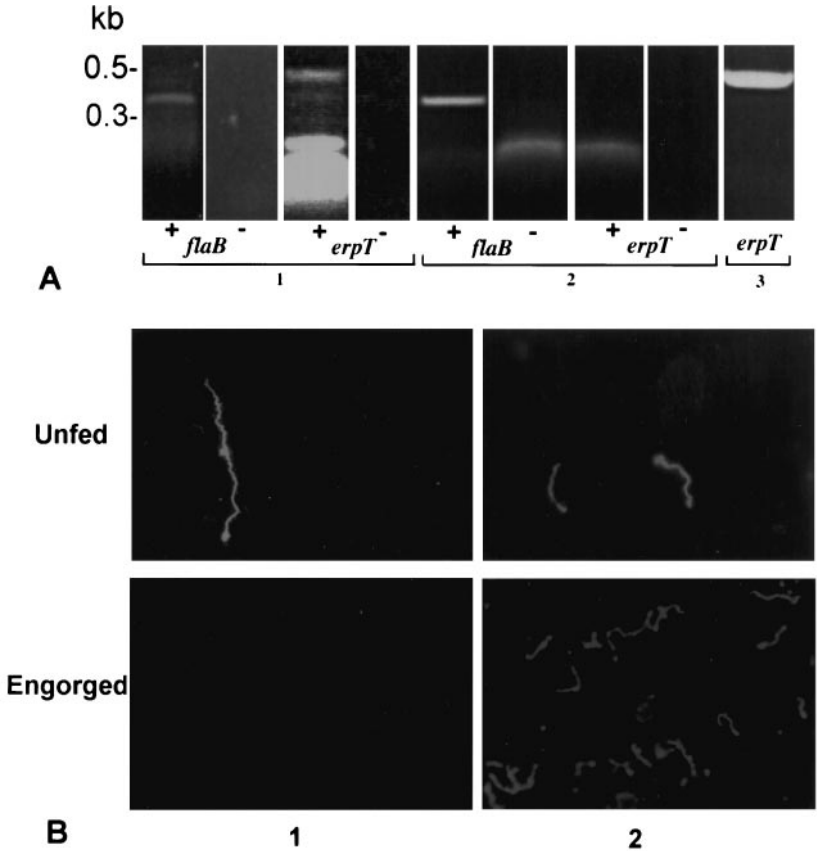

Fig. 2. *erpT* expression in flat and engorged ticks.
A. Detection of *erpT* or *flaB* mRNA by RNA PCR in unfed and fed *I. scapularis* nymphs. 1, Unfed ticks; 2, engorged ticks; 3, *Borrelia burgdorferi* DNA (control). With (+) or without (−) reverse transcriptase.
B. Immunofluorescence of *B. burgdorferi* in unfed or engorged ticks probed with ErpT antisera (1) or *B. burgdorferi* antisera (2).

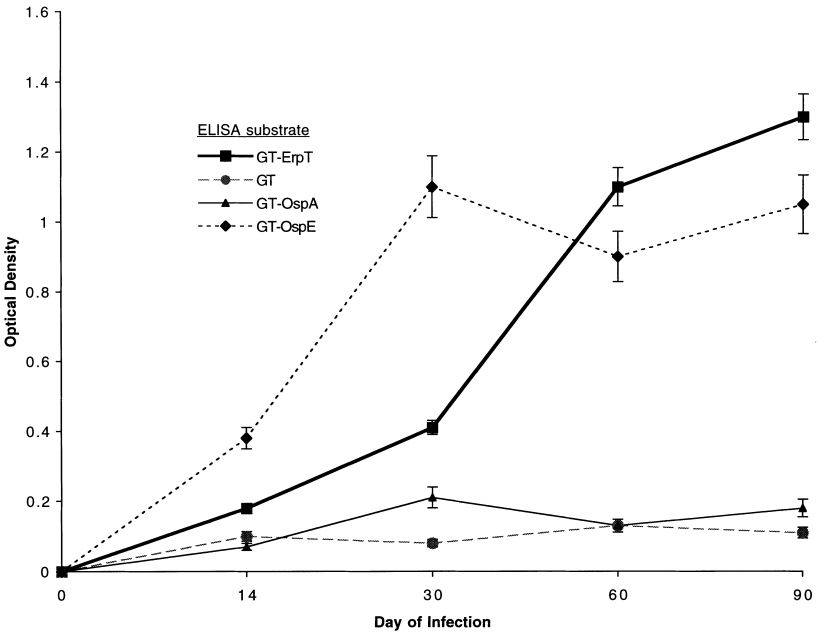

Fig. 3. ErpT antibody response during murine Lyme borreliosis. At 14, 30, 60 and 90 days, sera from five *Borrelia burgdorferi*-infected mice were pooled and examined for ErpT-specific antibodies using a recombinant GT–ErpT-based IgG ELISA. Recombinant GT (negative control), GT–OspA and GT–OspE were also used as antigens in ELISA.

ErpT elicits antibodies during Lyme borreliosis in mice but not humans

The humoral response to ErpT was assessed in murine and human Lyme borreliosis using an ELISA with recombinant GT–ErpT as the substrate. Sera from mice inoculated with 10^4 *B. burgdorferi* were collected up to 90 days after challenge. IgG to ErpT could be detected 30 days after infection and then increased in titre over the next 2 months (Fig. 3). As expected, OspE antibodies developed early in murine infection (Das *et al*., 1997), and OspA antibodies were not readily apparent. These data show that ErpT antibodies develop during persistent murine infection. Sera from 20 patients with well-documented early- and late-stage Lyme disease were examined for ErpT antibodies. The patients had erythema migrans, facial palsy, carditis manifested by conduction system abnormalities or Lyme arthritis. All the sera tested had high titre *B. burgdorferi* antibodies to *B. burgdorferi* whole-cell lysates by ELISA. Only 2 of the 20 sera tested, however, had detectable ErpT antibodies in the recombinant GT–ErpT-based ELISA (patient sera O.D $\geq$ 0.6, normal human sera OD $\leq$ 0.15). One of the two patients with ErpT IgG had Lyme carditis, and the other had Lyme arthritis. ErpT IgM was not detected in any of the patients.

Immunization studies in tick-borne disease

The role of ErpT-specific immune responses in immunity was investigated because *erpT* appears to be induced during infection. Mice immunized with recombinant ErpT developed high-titre ErpT antibodies, detectable at a sera dilution of 1:5000 on immunoblotting. Hyperimmune ErpT antisera does not bind OspE, OspF or OspE/F paralogues (Erps), such as recombinant P21 (not shown), suggesting that ErpT is a distant member of the Erp family (Stevenson *et al*., 1996; 1998; Fraser *et al*., 1997). ErpT-immunized mice were then challenged with an intradermal inoculation of 10^4 *B. burgdorferi* or by allowing five *I. scapularis* nymphs that were infected with *B. burgdorferi* to engorge upon mice. In either study, experimental syringe challenge, or tick-borne spirochaete transmission, the ErpT-immunized mice were not protected from infection (Table 2). Furthermore the severity of tibiotarsal arthritis was similar in ErpT-immunized mice (1.8 mean $\pm$ 0.4 standard deviation) and control animals (1.7 mean $\pm$ 0.6 standard deviation). Ticks that had engorged upon ErpT-immunized or control mice both had large numbers of spirochaetes (224 mean $\pm$ 86 standard deviation versus 204 mean $\pm$ 44 standard deviation) per high-power field by IFA, indicating that *B. burgdorferi* were not destroyed within ticks that had engorged upon ErpT-immunized mice.

Discussion

Borrelia burgdorferi inhabits various environments during its life cycle (Steere, 1989). Larval ticks acquire spirochaetes from infected mice. *Borrelia burgdorferi* persists through the moult and then resides in the gut lumen of flat nymphs. When nymphs engorge on a mammal, the spirochaetes migrate to the tick salivary glands and are then transmitted to the host. During mammalian infection, spirochaetes reside in different tissues including the skin, joints, heart and nervous system, among others. In

Table 2. *Borrelia burgdorferi* infection in ErpT-immunized mice.

		Evidence of murine Lyme borreliosis			
Immunogen	Challenge	Culture-positive	Arthritis	Carditis	Infection
ErpT	10^4 inocula	8/9	7/9	8/9	8/9
	Ticks	6/10	7/10	7/10	8/10
GT (control)	10^4 inocula	9/10	9/10	8/10	9/10
	Ticks	7/10	6/10	8/10	8/10

Mice were challenged with inocula of 10^4 *B. burgdorferi*, or by allowing five *B. burgdorferi*-infected *I. scapularis* nymphs to feed to repletion. After being killed, blood, urinary bladder, spleen and skin punches were cultured in BSK II medium for 2 weeks and examined by dark-field microscopy for spirochaetes. The tibiotarsal joints 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.

addition to these natural environments, *B. burgdorferi* can be cultivated in the laboratory medium, which, despite being an artificial milieu, represents a condition in which research regarding *B. burgdorferi* gene expression is conducted. We, and others, have demonstrated that *B. burgdorferi* can alter the expression of several genes depending on the surroundings, including *ospA*, *ospC*, *erps*, *lp6.6*, *eppa*, *p35* and *p37* (Champion *et al*., 1994; Schwan *et al*., 1995; Stevenson *et al*., 1995; 1998; Akins *et al*., 1996; de Silva *et al*., 1996; Coleman *et al*., 1997; Das *et al*., 1997; Fikrig *et al*., 1997; Lahdenne *et al*., 1997). We have now described a plasmid-encoded *B. burgdorferi* gene, *erpT*, that is expressed during murine infection and characterizes the expression of *erpT* in the vertebrate, in the vector and *in vitro*. Although *erpT* has 54% identity with *erpD* (Stevenson *et al*., 1996; Fraser *et al*., 1997), it is not a close relative of the *erp* gene family because an *erpT* probe does not recognize *ospE/F* on pulsed-field gene electrophoresis, and ErpT antiserum does not bind recombinant or native OspE or OspF. Erp expression has also been shown to be temperature dependent (Stevenson *et al*., 1995; 1998), however preliminary studies suggest that *in vitro erpT* expression is not influenced by temperature (not shown).

erpT was identified by selectively screening a *B. burgdorferi* genomic expression library with immune and hyperimmune sera. The differential screening suggested that *erpT* was not expressed *in vitro* but was expressed during murine infection, similar to what we have previously observed for *p21*, *p35* and *p37* (Das *et al*., 1997; Fikrig *et al*., 1997). Further immunological and molecular characterization of *erpT* demonstrated, however, that low levels of *erpT* mRNA can be detected in *B. burgdorferi* cultured in BSK II medium by RNA PCR. These small quantities of mRNA cannot be detected by Northern blotting, and ErpT antigen could not be identified by immunoblotting or IFA. Therefore, *erpT* is not readily expressed by *B. burgdorferi in vitro*, and the lack of sufficient ErpT antigen in cultured *B. burgdorferi* may account for the lack of detectable ErpT antibodies in hyperimmune sera.

The expression of *erpT* was extensively investigated in both the vector and the vertebrate because these environments reflect natural conditions in which spirochaetes reside. *erpT* was not abundantly expressed in ticks. Some *erpT* mRNA could be identified on *B. burgdorferi* within unfed nymphs by RNA PCR. ErpT antigen could also only be detected in a small minority (2.2%) of the spirochaetes within flat ticks by IFA. Within engorged ticks *erpT* mRNA, or ErpT antigen, could not be detected, despite the exponential increase in the number of *B. burgdorferi* in response to the blood meal (de Silva and Fikrig, 1995). Therefore, within flat ticks, as in BSK II medium *in vitro*, *erpT* exhibits low-level expression. Then, *B. burgdorferi erpT* was not expressed as spirochaetes were transmitted from engorged ticks to the mammalian host. During the early stages (14 days) of murine infection *erpT* mRNA was also not discernible and ErpT antibodies were not apparent. Then, as *B. burgdorferi* persisted in the mouse, *erpT* mRNA became perceptible and ErpT antibodies arose. Spirochaete numbers decreased during persistent murine infection. Thus, the ability to detect *erpT* mRNA at 30 days is not due to the number of spirochaetes in the tissue, but rather the induction of *erpT* expression. *erpT* is therefore a gene that is not readily expressed in the vector or during early murine infection, and is then induced during chronic infection in mice.

Borrelia burgdorferi gene expression is not only dependent upon the general environment, i.e. vector or mammalian host, but also on the location within the arthropod or host. For example, *ospA* is expressed by *B. burgdorferi* within tick guts but not within salivary glands of engorged ticks, indicating that *B. burgdorferi* gene expression differs in various sites within the vector (de Silva *et al*., 1996; Coleman *et al*., 1997). *p35* and *p37* are induced during vertebrate infection, however these genes are not expressed by the spirochaete at the initial site of skin inoculation, and are then induced as *B. burgdorferi* establish infection and disseminate to the spleen, joints, heart and distant skin sites (Fikrig *et al*., 1997). *erpT* is not expressed in the early stages of murine infection, including day 14, at which

time *B. burgdorferi* have disseminated to various organs, such as the joints and heart. After one month of infection, however, *B. burgdorferi* express *erpT* in all the tissues examined, including skin, joints, heart, bladder and spleen. At later stages of infection, including 2 and 3 months, *B. burgdorferi* continues to express *erpT*, which can be detected in several sites including the joints, heart and bladder. At these later time points, *erpT* mRNA could not be found in the skin despite the presence of comparable numbers of organisms in the joints, heart and skin. Moreover, *flaB* mRNA could be amplified from the skin at these intervals, indicating that the spirochaetes were present in all sites and intervals. *ospA* mRNA could only be detected at early time points of infection, consistent with studies indicating that *ospA* is repressed in the murine host (Montgomery *et al*., 1996). Therefore, *B. burgdorferi erpT* may be selectively downregulated in the skin during persistent murine infection. The expression of specific *B. burgdorferi* genes in the mammalian host, as in the vector, may be dependent upon the location in which *B. burgdorferi* resides, as well as the time point after infection.

Borrelia burgdorferi ErpT elicited strong humoral responses in mice. IgG was readily detectable at 30 days and persisted. The temporal development of ErpT antibodies correlated with the delayed appearance of *erpT* mRNA during infection. Surprisingly, the vast majority of the patients examined did not have ErpT-specific humoral responses. Most *B. burgdorferi* antigens, including those that are synthesized both *in vitro* and *in vivo*, and those that are selectively induced *in vivo*, have shown somewhat similar antibody responses in mice and humans (Fikrig *et al*., 1992a; 1994; 1997; Barthold and Bockenstedt, 1993; Dressler *et al*., 1993; Barthold *et al*., 1995; 1996). This suggests that ErpT may not be antigenic in humans, or that ErpT may be expressed during murine infection but not during human Lyme disease. The relative lack of *ospA* expression and OspA antibody production in mice also indicates that the results in murine Lyme borreliosis may not always be directly applied to human Lyme disease. Some patients with chronic Lyme arthritis develop high titre OspA antibodies, indicating that *ospA* can be expressed during persistent infection (Kalish *et al*., 1993). Studies focused on examining *erpT* expression in humans and other mammalian hosts will be able to address the issue of host-specific gene expression and antigenicity.

The role of the response to ErpT in protective immunity or disease modulation was examined because several *in vivo* synthesized antigens can influence the course of experimental murine infection. P35 and P37 antibodies, when administered to immunocompetent mice 24 h after *B. burgdorferi* challenge, have the ability to abort the infection (Fikrig *et al*., 1997). Moreover, OspC antibodies, when passively administered to persistently infection SCID mice, induce the regression of arthritis and clear the spirochaetes from the host (Zhong *et al*., 1997). Other studies, however, on the role of OspC in mediating protection have given conflicting results on protection from disease and did not show resolution of arthritis (Bockenstedt *et al*., 1998). Immunization with ErpT does not protect mice from infection or affect the severity of arthritis. One of several potential reasons for the lack of protective immunity is that not all the spirochaetes *in vivo* express *erpT*, as we have demonstrated in unfed ticks and in the murine skin during persistent infection, and therefore antibodies should not be able to eradicate these organisms.

Borrelia burgdorferi erpT is not readily expressed in ticks and is then induced during murine infection. During persistent infection, *erpT* expression may be most apparent in extracutaneous sites. These studies demonstrate the *B. burgdorferi* gene expression is remarkably diverse in both the vector and the vertebrate host, and that expression within the murine host may depend upon the time point, and specific tissue locations that are examined. Efforts directed at delineating the signals that influence *B. burgdorferi* gene expression *in vitro*, in the vector, in the vertebrate host and in specific tissues, as well as the function of selectively expressed genes, will help facilitate our understanding of the pathogenesis of Lyme borreliosis.

Experimental procedures

Borrelia burgdorferi

A clonal isolate of *B. burgdorferi* N40 (cN40) that is infectious and pathogenic in mice was used (Barthold *et al*., 1993). Spirochaetes were cultivated in BSK II medium at 33°C (Barbour, 1984).

Mice

Three-week-old female C3H/HeNCr (C3H) mice from the Frederick Cancer Research Center, Frederick, MD, USA, were housed in filter frame cages. Mice were euthanized with CO_2.

Antisera and library screening

Sera were recovered from mice with culture-verified infection after intradermal inoculation with 10^2 *B. burgdorferi* at 30 days after inoculation (immune sera) or from mice hyperimmunized (hyperimmune sera) with heat-killed spirochaete lysates in complete Freund's adjuvant (CFA) (Suk *et al*., 1995). A lambda ZAP II genomic expression library of *B. burgdorferi* N40 was probed with immune and hyperimmune sera (1:100 dilution in phosphate-buffered saline) as described (Suk *et al*., 1995). Goat anti-mouse Ig conjugated to alkaline phosphatase was used as the secondary antibody and detected by incubation with nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl-phosphate (BCIP). Plaques that only reacted with immune sera were isolated. The pBluescript plasmids were

then excised using the R408 helper phage. The nucleotide sequences were determined using the Circumvent Thermal Cycle Dideoxy DNA sequencing kit (New England Biolabs). The gene was designated *erpT*, based on the identified open reading frame.

Pulsed-field gel electrophoresis

For pulsed-field gel electrophoresis, DNA plugs containing 10^5 *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-DRII system (Bio-Rad Laboratories) (Lam *et al*., 1994). The gel was run at 14°C for 18 h at 198 V with ramped pulse times from 1 to 30 s. Southern blotting was performed with a ^{32}P-labelled *erpT* or *ospA* probe using the Prime-It random primer kit (Stratagene).

Northern blot analysis

Fifteen micrograms of RNA from cultured *B. burgdorferi* 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 h at 42°C, and then hybridized using the same conditions with an *erpT* or *ospA* probe overnight. The membranes were washed in 0.1× SSC and 0.1% SDS at 55°C before development of film.

PCR

PCR amplification of *B. burgdorferi* DNA was performed for 30 cycles with denaturing, annealing and extension temperatures of 94°C for 1 min, 55°C for 1 min and 72°C for 2 min 5′ and 3′ primers were: 5′-TCTGACGATCTAGGTCAAACCACA-3′; 5′-CCCTCTAATTTGGTGCCATTTG-3′ for *ospA*; 5′-CAATCAGGTAACGGC-3′ and 5′-TCTATTTTGGAAAGC-3′ for *flaB*; and 5′-TTCACAAGTAGCA CAACAT GCTCC-3′ and 5′-GCTATCACCACCA ATTTCAAGTCC-3′ for *erpT*. For RNA PCR, total RNA was isolated from the spleens, skin, tibiotarsi and hearts of *B. burgdorferi*-infected mice or from spirochaetes cultured in BSK II medium by acid guanidinium thiocyanate phenol–chloroform extraction (Suk *et al*., 1995). RNA was treated with RNase-free DNase (Promega) for 3 h at 37°C. A 2 μg sample of RNA was then reverse transcribed using rTth DNA polymerase (Perkin Elmer). PCR was then carried out for 30 cycles.

Serial dilution PCR was used to quantify *B. burgdorferi* in specific tissues, as previously described (Yang *et al*., 1994; Anguita *et al*., 1996). The sensitivity of the *flaB* and *erpT* primers was first determined using DNA from 10^6 *B. burgdorferi* cultured in BSK II medium. Serial dilutions of the DNA from laboratory cultured spirochaetes was used as the template for PCR. An amplified PCR product could be readily detected when DNA corresponding to 10–100 spirochaetes was used as the template, consistent with published reports (Yang *et al*., 1994; Anguita *et al*., 1996). Serial dilution PCR was then performed using DNA extracted from tissues of *B. burgdorferi*-infected mice. A 1 mg sample of extracted DNA from each tissue was used as the initial template for PCR, which was the subjected to serial 10-fold dilutions. When the dilution at which a signal could no longer be detected was identified, then serial twofold dilutions were performed around that point. In this manner, the spirochaete/tissue DNA could be calculated at selected intervals. For quantitative RNA PCR, a competition assay was performed, based on established techniques (Reiner *et al*., 1993). Competitor constructs were created in which a 540 bp fragment of unrelated DNA (a fragment of the gene encoding the 44 kDa antigen of the agent of human granulocytic ehrlichiosis) was flanked by the *erpT* primers. Varying concentrations of competitor were added to the RNA PCR reactions, and the concentration at which the amplified competitor product and the gene of interest (*erpT*) were similar in intensity was used to calculate the quantity of mRNA.

Protein purification and ErpT-antisera production

Recombinant ErpT was expressed as a fusion protein with glutathione transferase (GT). The *erpT* gene was subcloned into pMX, a modified pGEX-2T vector (Sears *et al*., 1991). The ligation mixture was used to transform *Escherichia coli* DH5α, and colonies containing the recombinant plasmid were isolated on Luria broth supplemented with ampicillin.

Recombinant GT–ErpT fusion protein was induced by growing the bacteria to logarithmic phase and adding 1 mM isopropyl-1-thio-beta-galactoside (IPTG) for 3 h (Fikrig *et al*., 1997). The *E. coli* was placed in PBS with 1% Triton and subjected to sonication. The cell supernatant was separated by centrifugation at 1000 × *g* for 8 min and GT–ErpT was purified by passing the supernatant over a glutathione column. GT–ErpT was eluted from the column using a solution containing excess glutathione. Recombinant GT (control) was prepared in an identical fashion. In some cases, GT was cleaved from the GT–ErpT fusion protein with thrombin, using a thrombin cleavage site that exists between GT and ErpT. Recombinant ErpT (without the GT-fusion) was used as a substrate in dot-blots that were probed with GT–ErpT antisera, to demonstrate the presence of ErpT-specific antibodies (Fig. 1B, lanes 3 and 4).

ErpT-antiserum was prepared by hyperimmunizing mice or rabbits with 10 or 50 μg, respectively, of GT–ErpT in CFA and boosting the animals twice over a period of 1 month with the same amount of antigen in IFA. GT antiserum (control) was prepared in a similar manner.

ELISA and immunoblots

For ELISA, triplicate sets of 96-well microtitre plates were coated with 200 ng (1 μg ml^{-1}, 200 μl per well) of *B. burgdorferi* lysates, GT, GT–OspA, GT–OspE or GT–ErpT and incubated overnight at 4°C. Plates were washed three times with PBS with 0.05% Tween (PBST). Triplicate samples of patient or murine sera (200 μl per well, diluted 1:100) were added to the plates and incubated for 1 h. Plates were washed three times with PBST. Goat anti-human or anti-mouse IgM and IgG diluted 1:1000 and linked to alkaline phosphatase were added to each well. Plates were incubated at room temperature for 1 h and washed three times with PBST. *p*-Nitrophenol phosphate (200 μl) (1 mg ml^{-1} in glycine buffer, pH 10.5) was added to each well and monitored at 405 nm. The reaction was stopped with 3 M NaOH.

For immunoblotting, 5 μg of *B. burgdorferi* whole-cell lysates were resolved in 12% minigels by SDS–PAGE and transferred to nitrocellulose membranes. The nitrocellulose strips were then probed with antisera (diluted 1:100). The secondary antibody was alkaline phosphatase conjugated goat anti-mouse or anti-human Ig. Colour development was performed using NBT and BCIP. For dot-blotting, 5 μg of recombinant ErpT was directly placed onto nitrocellulose, and then probed in an identical fashion.

Infection and immunization studies

For infection, 4-week-old C3H mice were intradermally challenged with 10^4 *B. burgdorferi* N40 and killed at 14, 30, 60 and 90 days. In a single experiment, groups of 20 mice were inoculated with *B. burgdorferi* and four mice were killed at each of the four time points. The experiment was repeated three times. In each individual experiment, samples from the four mice killed at each interval were pooled before RNA extraction.

Mice were subcutaneously hyperimmunized with 10 μg of GT–ErpT in CFA and boosted at 14 and 28 days with the same amount of antigen in IFA, according to published protocols (Fikrig *et al*., 1990). Control mice were hyperimmunized with GT in an identical fashion. Fourteen days after the last boost, sera from the hyperimmunized animals were used to probe recombinant ErpT (prepared without the GT fusion partner using thrombin cleavage) to determine the titre of specific antibody. Hyperimmunized mice were challenged 14 days after the last boost with intradermal inoculations of 10^4 spirochaetes or by allowing five *B. burgdorferi*-infected ticks to feed on individual mice (Fikrig *et al*., 1990; Fikrig *et al*., 1992b). Mice were killed at 14 days. At necropsy, blood, spleen, urinary bladder and skin specimens were collected aseptically, cultured in BSK II medium, and examined by dark-field microscopy for spirochaetes. The tibiotarsal joints and the hearts were formalin fixed, paraffin embedded, sectioned and examined microscopically for inflammation (Barthold *et al*., 1991). Arthritis was graded by blinded analysis on a scale from 0 (no inflammation) to 3 (severe) as described (Fikrig *et al*., 1993).

Examination of B. burgdorferi *by immunofluorescence*

Groups of *B. burgdorferi* N40-infected *I. scapularis* nymphs, including unfed ticks and ticks fed to repletion on mice, were checked for ErpT on spirochaetes within the vector by indirect IFA. Each tick was lightly homogenized in 100 μl of PBS, and 10 μl aliquots were spotted on a silylated glass slide. The slides were air dried and fixed with 4% paraformaldehyde. The specimens were incubated in ErpT antisera (1: 100 dilution) for 1 h, washed, followed by incubation in anti-mouse IgG coupled with FITC (1: 500 dilution) for another hour, and viewed under a Zeiss Axioskop fluorescence microscope. *Borrelia burgdorferi* antisera, which recognizes *B. burgdorferi* within unfed and engorged ticks, was used as a positive control. GT antiserum was used as the negative control. Spirochaetes cultured in BSK II medium were examined by IFA in an identical fashion. A total of 50–120 high-power fields were examined.

In the immunization studies, engorged ticks fell from the mice within 4 days and were collected in water baths below the cages. To quantify *B. burgdorferi*, 10 μl aliquots of tick homogenate was spotted on a clean slide, air dried and then fixed in cold acetone for 10 min. The slides were incubated with fluorescein isothiocyanate (FITC)-labelled rabbit *B. burgdorferi* N40 antiserum, mounted under a coverslip and examined on a Zeiss Axioskop fluorescence microscope.

Acknowledgements

This work was supported by grants from the National Institutes of Health (AI-45253, AI-37993, AI-26815 and AI-49387), Centers for Disease Control (U50-CCU106581), Arthritis Foundations, and American Heart Association. Erol Fikrig is the recipient of a Burroughs Wellcome Clinical Scientist Award in Translational Research. Richard A. Flavell is an Investigator of the Howard Hughes Medical Institute.

References

Akins, D., Porcella, S.R., Popova, T.G., Shevchenko, D., Baker, S.I., Li, M., Norgard, M.V., and Radolf, J.D. (1996) Evidence for *in vivo* but not *in vitro* expression of a *Borrelia burgdorferi* outer surface protein F (OspF) homologue. *Mol Microbiol* **18:** 507–520.

Anguita, J., Persing, D.H., Rincon, M., Barthold, S.W., and Fikrig, E. (1996) Effect of anti-interleukin 12 treatment on murine Lyme borreliosis. *J Clin Invest* **97:** 1028–1034.

Barbour, A.G. (1984) Isolation and cultivation of Lyme disease spirochetes. *Yale J Biol Med* **57:** 521–525.

Barthold, S.W., and Bockenstedt, L.K. (1993) Passive immunizing activity of sera from mice infected with *Borrelia burgdorferi*. *Infect Immun* **61:** 4696–4702.

Barthold, S.W., Persing, D.H., Armstrong, A.L., and Peeples, R.A. (1991) Kinetics of *Borrelia burgdorferi*: Dissemination and evolution of disease following intradermal inoculation of mice. *Am J Pathol* **163:** 263–273.

Barthold, S.W., deSouza, M.S., Janotka, J.L., Smith, A.L., and Persing, D.H. (1993) Chronic Lyme borreliosis in the laboratory mouse. *Am J Pathol* **143:** 959–971.

Barthold, S.W., Fikrig, E., Bockenstedt, L.K., and Persing, D.H. (1995) Circumvention of outer surface protein A immunity by host-adapted *Borrelia burgdorferi*. *Infect Immun* **63:** 2255–2261.

Barthold, S., Souza, M.D. and Feng, S. (1996) Serum-mediated resolution of Lyme arthritis in mice. *Lab Invest* **74:** 57–67.

Barthold, S.W., Feng, S., Bockenstedt, L.K., Fikrig, E., and Feen, K. (1997) Protective and arthritis-resolving activity in serum of mice actively infected with *Borrelia burgdorferi*. *Clin Infect Dis* **25:** S9–S17.

Bockenstedt, L.K., Hodzic, E., Feng, S., de Bourrel, K.W.S., Ilva, A., Montgomery, R.R., *et al*. (1998) *Borrelia burgdorferi* strain-specific OspC-mediated immunity in mice. *Infect Immun* **65:** 4661–4667.

Champion, C.I., Blanco, D.R., Skare, J.T., Haake, D.A., Giladi, M., Foley, D., Miller, J.N., *et al*. (1994) A 9.0-kilobase-pair circular plasmid of *Borrelia burgdorferi* encodes an exported protein: evidence for expression only during infection. *Infect Immun* **62:** 2653–2661.

Coleman, J.L., Gebbia, J.A., Piesman, J., Degen, J.L., Bugge, T.H., and Benach, J.L. (1997) Plasminogen is required for

efficient dissemination of *B. burgdorferi* in ticks and for enhancement of spirochetemia in mice. *Cell* **89:** 1111–1119.

Das, S., Barthold, S.W., Giles, S.S., Montgomery, R.R., Telford, S.R., and Fikrig, E. (1997) Temporal pattern of *Borrelia burgdorferi p21* expression in ticks and the mammalian host. *J Clin Invest* **99:** 987–995.

Dressler, F., Whalen, J.A., Reinhardt, B.A., and Steere, A.C. (1993) Western blotting in the serologic diagnosis of Lyme disease. *J Infect Dis* **167:** 392–400.

Fikrig, E., Barthold, S.W., Kantor, F.S., and Flavell, R.A. (1990) Protection of mice against the Lyme disease agent by immunizing with recombinant OspA. *Science* **250:** 553–556.

Fikrig, E., Bockenstedt, L., Barthold, S.W., Chen, M., Ali-Salaam, P., Telford, S.R., and Flavell, R.A. (1994) Sera from patients with chronic Lyme disease protect mice from infection. *J Infect Dis* **169:** 568–575.

Fikrig, E., Huguenel, E.D., Berland, R., Rahn, D.W., Hardin, J.A., and Flavell, R.A. (1992a) Serologic diagnosis of Lyme disease using recombinant outer surface proteins A and B and flagellin. *J Infect Dis* **165:** 1127–1132.

Fikrig, E.F., Telford, S.R., Barthold, S.W., Kantor, F.S., Spielman, A., and Flavell, R.A. (1992b) Elimination of *Borrelia burgdorferi* from vector ticks feeding on OspA-immunized mice. *Proc Natl Acad Sci USA* **89:** 5418–5421.

Fikrig, E., Barthold, S.W., and Flavell, R.A. (1993) OspA vaccination of mice with established *Borrelia burgdorferi* infection alters disease but not infection. *Infect Immun* **61:** 2553–2557.

Fikrig, E., Barthold, S.W., Sun, W., Feng, W., Telford, S.R., and Flavell, R.A. (1997) *Borrelia burgdorferi* P35 and P37 proteins, expressed in vivo, elicit protective immunity. *Immunity* **6:** 531–539.

Fraser, C.M., Casjens, S., Huang, W.M., Sutton, G.G., Clayton, R., Lathigra, R., *et al*. (1997) Genomic sequence of a Lyme disease spirochaete, *Borrelia burgdorferi*. *Nature* **390:** 580–587.

Fuchs, H., Wallich, R., Simon, M.M., and Kramer, M.D. (1994) The outer surface protein A of the spirochete *Borrelia burgdorferi* is a plasmin (ogen) receptor. *Proc Natl Acad Sci USA* **91:** 12594–12598.

Kalish, R., Leong, J.M., and Steere, A.C. (1993) Association of treatment-resistant chronic Lyme arthritis with antibody reactivity to OspA and OspB of *Borrelia burgdorferi*. *Infect Immun* **61:** 2774–2779.

Lahdenne, P., Porcella, S.F., Hagman, K.E., Akins, D.R., Popova, T.G., Cox, D.L., *et al*. (1997) Molecular characterization of a 6.6-kilodalton *Borrelia burgdorferi* outer membrane-associated lipoprotein (lp6.6) which appears to be downregulated during mammalian infection. *Infect Immun* **65:** 412–421.

Lam, T.T., Nguyen, T.P., Montgomery, R.R., Kantor, F.S., Fikrig, E., and Flavell, R.A. (1994) Outer surface proteins E and F of *Borrelia burgdorferi*, the agent of Lyme disease. *Infect Immun* **62:** 290–298.

Montgomery, R.R., Malawista, S.E., Feen, K.J., and Bockenstedt, L.K. (1996) Direct demonstration of antigenic substitution of *Borrelia burgdorferi* ex vivo: exploration of the paradox of the early immune response to outer surface proteins A and C in Lyme disease. *J Exp Med* **183:** 261–269.

Nguyen, T.P., Lam, T.T., Barthold, S.W., Telford, S.R., Flavell, R.A., and Fikrig, E. (1994) Partial destruction of *Borrelia burgdorferi* within ticks that engorged on OspE- or OspF-immunized mice. *Infect Immun* **62:** 2079–2084.

Reiner, S.L., Zheng, S., Corry, D.B., and Locksley, R.M. (1993) Constructing polycompetitor cDNAs for quantitative PCR. *J Immunol Methods* **165:** 37–46.

Schwan, T.G., Piesman, J., Golde, W.T., Dolan, M.C., and Rosa, P.A. (1995) Induction of an outer surface protein on *Borrelia burgdorferi* during tick feeding. *Proc Natl Acad Sci USA* **92:** 2909–2913.

Sears, J., Fikrig, E., Nakagawa, T., Deponte, K., Marcantonio, N., Kantor, F., and Flavell, R.A. (1991) Molecular mapping of OspA-mediated protection against *Borrelia burgdorferi*, the Lyme disease agent. *J Immunol* **147:** 1995–2001.

de Silva, A.M., and Fikrig, E. (1995) Growth and migration of *Borrelia burgdorferi* in Ixodes ticks during blood feeding. *Am J Trop Med Hyg* **53:** 397–404.

de Silva, A.M., Telford, S.R., Brunet, L.R., Barthold, S.W., and Fikrig, E. (1996) *Borrelia burgdorferi* OspA is an arthropod-specific transmission-blocking Lyme disease vaccine. *J Exp Med* **183:** 271–275.

de Silva, A.M., Fish, D., Burkot, T.R., Zhang, Y., and Fikrig, E. (1997) OspA antibodies inhibit the acquisition of *Borrelia burgdorferi* by Ixodes ticks. *Infect Immun* **65:** 3146–3150.

Steere, A.C. (1989) Lyme disease. *N Engl J Med* **321:** 586–594.

Stevenson, B., Schwan, T.G., and Rosa, P.A. (1995) Temperature-related differential expression of antigens in the Lyme disease spirochete, *Borrelia burgdorferi*. *Infect Immun* **63:** 4535–4539.

Stevenson, B., Tilly, K., and Rosa, P.A. (1996) A family of genes located on four separate 32-kilobase circular plasmids in *Borrelia burgdorferi* B31. *J Bacteriol* **178:** 3508–3516.

Stevenson, B., Bono, J.L., Schwan, T.G., and Rosa, P. (1998) *Borrelia burgdorferi* Erp proteins are immunogenic in mammals infected by tick-bite, and their synthesis is inducible in cultured bacteria. *Infect Immun* **66:** 2648–2654.

Suk, K., Das, S., Sun, W., Jwang, B., Barthold, S.W., Flavell, R.A., and Fikrig, E. (1995) *Borrelia burgdorferi* genes selectively expressed in the infected host. *Proc Natl Acad Sci USA* **92:** 4269–4273.

Yang, L.M., Weis, J.H., Eichwald, E., Kolbert, C.P., Persing, D.H., and Weis, J.J. (1994) Heritable susceptibility to severe *Borrelia burgdorferi*-induced arthritis is dominant and is associated with persistence of large numbers of spirochetes in tissues. *Infect Immun* **62:** 492–500.

Zhang, J.R., Hardham, J.M., Barbour, A.G., and Norris, S.J. (1997) Antigenic variation in Lyme disease Borreliae by promiscuous recombination of Vmp-like sequence cassettes. *Cell* **89:** 275–285.

Zhong, W.M., Stehle, T., Museteanu, C., Siebers, A., Gern, L., Kramer, M., Wallich, R., and Simon, M.M. (1997) Therapeutic passive vaccination against chronic Lyme disease in mice. *Proc Natl Acad Sci USA* **94:** 12533–12538.