COMPARATIVE STUDIES ON IMMUNE RESPONSES TO TICK TRANSMITTED AND NEEDLE INOCULATED INFECTION OF *BORRELIA BURGDORFERI* IN BALB/C MICE

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Submitted to the Faculty of the
Graduate College of the
Oklahoma State University
in partial fulfillment of
the requirements for
the Degree of
DOCTOR OF PHILOSOPHY
December, 1997

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ACKNOWLEDGMENTS

I wish to express my sincere appreciation and thanks to my major advisor, Dr. Stephen K. Wikel for his critical supervision, constructive guidance, inspiration, and constant encouragement throughout this study. The participation and invaluable help of my other committee members including Dr. John Sauer, Dr. Jack Dillwith, Dr. Kim Burnham, and Dr. Russel Wright is gratefully acknowledged. I would also like to thank Dr. R.N. Ramachandra and Dr. Douglas Bergman for their help and extremely useful discussions on various aspects of this study.

The companionship and support of my friends and colleagues, too numerous to name here, during my stay in Stillwater, OK was invaluable. A special thanks is extended to my friend and roommate Dr. Karamjeet Pandher for his moral support and affection.

I thank Vladimir Beregvoy for providing me with adult *Ixodes scapularis* ticks and Dr. A.G. Barbour at University of Texas Health Center for supplying me with a mutant strain of *Borrelia burgdorferi* used in this study.

I express my extreme indebtedness to my parents, brother, and sister-in-law for their selfless love and sacrifice which served as a constant source of encouragement to succeed in the face of adversity.

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NOMENCLATURE

ADP	adenosine diphosphate
BLOTTO	Bovine Lacto Transfer Technique
BSA	bovine serum albumin
BSK II	Barbour-Stoenner-Kelly II medium
CDC	Centers for Disease Control
$CD4^+$	cells positive for cluster of differentiation designation 4 (helper T-lymphocytes)
cm	centimeter
CMI	cell mediated immunity
CNS	central nervous system
Con A	concanavalin-A
CPM	counts per minute
DNA	deoxyribonucleic acid
ECM	erythema chronicum migrans
ELISA	enzyme-linked immunosorbant assay
HLA	human leukocyte antigens
hr(s)	hour(s)
HRPO	horse radish peroxidase
HSP	heat shock protein

IFA	indirect fluorescent antibody test
IFN-γ	interferon-γ
IL	interleukin
IP	intraperitoneal
kDa	kilodalton
LPS	lipopolysaccharide
Osp	outer surface protein
PBS	phosphate buffered saline
PHA	phytohemagglutinin
PGE_2	prostaglandin E_2
RNA	ribonucleic acid
SCID	severe combined immunodeficiency
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
S.E.	standard error
S.I.	stimulation index
TNF-α	tumor necrosis factor-α
WSE	whole spirochete extract

CHAPTER I

PERSPECTIVES AND OVERVIEW

Introduction

Lyme borreliosis is the most commonly reported arthropod-borne infection of humans in the United States (CDC, 1993; Spach *et al.*, 1993). In the United States, this malady was first described by Steere *et al.*, (1977) who reported an epidemic, initially suspected to be juvenile rheumatoid arthritis, in the children of three communities in and around Lyme, Connecticut. However, geographical clustering of cases and presence of a skin rash in some of the patients was suggestive of an arthropod borne infection (Steere *et al.*, 1978a, Steere *et al.*, 1978b). These observations were confirmed and it was found that the causative agent of Lyme borreliosis is a novel spirochete, *Borrelia burgdorferi*, which is transmitted to the vertebrate host following the bite of infected ixodid ticks (Burgdorfer *et al.*, 1982, Barbour *et al.,* 1983a and b, Johnson *et al.*, 1984a). Tick vectors include *Ixodes ricinus* and *Ixodes persulcatus* in Europe, *Ixodes pacificus* in the western US, and *Ixodes scapularis* in the Eastern and Midwestern US (Sigal and Curran, 1991). The tick originally described as *I. dammini* has been determined to be conspecific with *I. scapularis* (Oliver *et al.*, 1993).

A total of 40,195 cases of Lyme borreliosis were reported to the Centers for Disease Control from 1982 to 1991 (CDC, 1993). In 1992, Lyme borreliosis accounted for more than 90 % of all vector-borne illnesses reported in the United States with 45 states reporting at least one case (CDC, 1993). The case density is highest in the coastal northeast between Maryland and Massachusetts, in the upper Midwest in Wisconsin and

Minnesota, and in the West in California and Oregon (CDC, 1993). In Europe, Lyme borreliosis has been reported from Austria, France, Germany, Sweden, Switzerland, and Russia; and in Asia from China, Japan, and Siberia (Nocton and Steere, 1995).

In the United States, the larvae of vector tick *Ixodes* sp. usually get infected after feeding on *B. burgdorferi* infected white-footed mouse *Peromyscus leucopus* during late summer (Levine *et al.*, 1985, Wilson and Spielman, 1985). The mouse acts as a reservoir host since it can remain spirochetemic without mounting an inflammatory response (Levine *et al.*, 1985). The spirochete stays in the gut of the replete larvae through out winter and the resulting infected nymph (transstadial transmission), takes its blood meal in late spring or early summer preferably from white-footed mice (Wilson and Spielman, 1985). This feeding can infect previously uninfected mice which serve as reservoirs for subsequent generation of larvae later in the summer, however, accidental attachment of these nymphs results in transmission of spirochetes to humans (Levine *et al.*, 1985). The nymphs have to remain attached to the host for at least 48-72 hours before the spirochete can be successfully transmitted (Ribeiro *et al.*, 1987). Adult ticks although capable of transmission of disease usually do not feed on humans and prefer white-tailed deer, *Odocoileus virginianus* (Wilson *et al.*, 1986).

Lyme borreliosis is a multisystem inflammatory disorder of humans and presents varying clinical picture during the course of infection (Steere, 1989). The pathognomonic initial skin rash called erythema chronicum migrans (ECM), arising after the infectious tick bite, is present or recalled by 50-70 % of patients. This early stage is characterized by symptoms such as fatigue, malaise, lethargy, headache, myalgia (muscle pain), arthralgia (joint pain), and regional/generalized lymphnadenopathy (Steere *et* al., 1978b; Sigal,

1992a). Several serological tests such as indirect fluorescent antibody test, enzyme linked immunosorbant assay, Western blotting combined with history and clinical picture can be used at this stage to confirm the diagnosis (Magnarelli, 1995). In the case of failure to diagnose and successfully treat the disease with antibiotics at this stage, the clinical course progresses to the second stage called early disseminated disease which can have associated neurologic symptoms (10-15 % of untreated patients) and cardiac manifestations (8 % of untreated patients) occurring within days to 10 months following tick bite (Sigal, 1992a). The third stage known as chronic disease has musculoskeletal manifestations which may not become apparent for months to years after initial infection (Steere *et al.*, 1987). Symptoms may sometimes persist or reappear after treatment, particularly in later stages of the disease, and sometimes patients may be refractory to therapy (Steere, 1989; Dattwyler and Halperin, 1987).

Development of a practical and efficacious vaccine for this disease is needed. An important aspect of research needed to achieve this goal is a basic understanding of pathogenesis of this disease, particularly the tick transmitted infection. Role of spirochetal antigens in inducing host cellular/humoral immune responses and in pathogenesis must be investigated in order to better understand the disease process and develop rational control strategies.

Convenient animal models such as rabbits (Burgdorfer, 1984), Syrian hamsters (Johnson *et al.*, 1984b); rats (Barthold *et al.*, 1988); immunodeficient mice (Schaible *et al.*, 1989); inbred mice (Barthold *et al.*, 1990); outbred mice (Masuzawa *et al.*, 1992); gerbils (Preac-Mursic *et al.*, 1992); guinea pigs (Sonnesyn *et al.*, 1993); dogs (Appel *et al.*, 1993); and Rhesus monkeys (Philipp *et al.*, 1993) have been developed to study

various aspects of host immune responses to *B. burgdorferi* infection and pathogenesis. Lyme borreliosis spirochetes and several of their outer surface proteins have been shown to induce protective antibody response in animals (Lim *et al.*, 1994). There is a growing interest in studies on effects of spirochetes and their antigens on the cellular immune pathways of hosts and their role in cell mediated immunity and pathogenesis of disease (Lim *et al.*, 1995a; Lim *et al.*, 1995b)

The vast majority of studies have used needle inoculation of culture grown *B. burgdorferi* to either initiate infection or to challenge vaccinated hosts (Roehrig *et al.*, 1992). Immune responses to needle inoculated infection differ considerably from the tick-transmitted infection (Roehrig *et al.*, 1992). Antibody responses to outer surface protein A (OspA, 31 kDa) and OspB (34 kDa) are produced late in human infection if at all, following the bite of an infected tick (Craft *et al.*, 1986; Habicht, 1988). Hamsters needle inoculated with either cultured organisms or infected tick homogenates readily produced a large amount of antibody to OspA and OspB, but not following the bite of an infected tick (Roehrig *et al.*, 1992).

The delayed immune response to OspA and OspB following the tick transmitted infection has not been adequately explained or explored, although, several hypotheses in this regard have been proposed. These include *B. burgdorferi* occupying an immune-privileged site in the host (Habicht, 1988), needle inoculation delivering a much larger antigenic load as compared to infected tick bite (Roehrig *et al.*, 1992), expression of lower amounts of OspA and OspB in the spirochetes while they are in the tick, and regulation by bacteria or tick in presentation of these antigens to host immune system so that they appear different from culture grown antigens (Roehrig *et al.*, 1992). More

recently, Schwan *et al.*, (1995) showed selective down regulation of OspA expression on spirochetes during tick feeding with concurrent upregulation of OspC. Complex interaction at the tick-host-pathogen interface are critical to the establishment of infection. Immunosuppressive and other pharmacological properties of tick saliva might affect the nature and magnitude of immune responses generated to the vector-borne pathogen (Wikel, 1996a).

Differences in the cell mediated immune responses following tick initiated versus needle inoculation induced infection have been ignored to date and no reports exist in the literature in this regard. With a possible role for *B. burgdorferi* specific T- lymphocytes in development of severe destructive arthritis in hamsters (Lim *et al.*, 1995a and b), it is important to investigate the differences in cell mediated immune responses to needle inoculated versus tick transmitted infection.

The working hypothesis of this study was that the nature and magnitude of humoral and cellular immune responses differs during tick transmitted infection as compared with infection established by needle inoculation of culture grown *B. burgdorferi*. BALB/c mice were infected by either tick-transmission or needle inoculation and the nature of humoral and cellular immune responses were determined at different time intervals during the course of infection. This study was designed as a comprehensive investigation into the qualitative and quantitative differences in humoral and cellular immune responses to *B. burgdorferi* infection in BALB/c mice initiated by either needle inoculation of culture grown spirochetes or following infestation with infected *I. scapularis* nymphs.

Comparative antibody responses following infection by either method were monitored both qualitatively and quantitatively. Enzyme linked immunosorbant assay (ELISA) and immunoblotting were used to monitor immunoglobulin isotype, antigen specificity and titer of *Borrelia*-specific antibodies in sera collected at different intervals following infection. An *in vitro B. burgdorferi* neutralization assay was used to screen sera collected at different intervals following infection for the presence of borreliacidal antibodies. Cellular immune responses to whole *B. burgdorferi* sonicate and defined molecular weight fractions obtained by preparative sodium dodecyl sulfate-poly acrylamide gel electrophoresis (SDS-PAGE) of whole *B. burgdorferi* sonicate were determined by *in vitro* proliferative responses of splenocytes collected from mice at different time periods after tick transmitted or needle inoculated infection.

Specific Aims:

1) Determine qualitative and quantitative differences in the humoral immune response mounted by BALB/c mice against *B. burgdorferi* infection established by needle inoculation of culture grown spirochetes or initiated by an infestation with infected *I. scapularis* nymphs.
    a) Determine the differences in immunoglobulin isotypes, their amounts, and the time of appearance during the course of infection using isotype specific ELISA (IgM and IgG) on the sera collected at different intervals following initiation of infection.
    b) Monitor the appearance of borreliacidal antibodies in the sera collected at different intervals following initiation of infection by either method using an *in vitro* spirochete neutralization assay.

2) Determine the differences in the cellular immune responses mounted by BALB/c mice against tick transmitted and needle inoculated *B. burgdorferi* infection. To collect splenocytes from mice infected by either method at different time intervals and to study their *in vitro* proliferative responses against

   a) whole *B. burgdorferi* extracts.

   b) isolated borrelial antigens obtained by preparative SDS-PAGE

Literature Review

A large body of literature on various aspects of the Lyme borreliosis has accumulated over the past 20 years since the recognition of this disease in North America in Lyme, Connecticut. (Steere *et al.*, 1977). A Medline search with key word "*Borrelia burgdorferi*" for the time period between January, 1996 through April 1997 resulted in 275 articles indicating the intense research activity related to this topic. The purpose of this review is not to list each and every study done to date, but to provide information on different aspects of this disease and show how the research described in this dissertation relates to and builds upon the published body of knowledge.

History and Etiology: In early 1920's, scientists in Europe recognized a characteristic expanding skin lesion called erythema chronicum migrans (ECM), which was related to the bite of the tick *Ixodes ricinus* (Afzelius, 1921* ; Lipschutz, 1923*). Before that, Herxheimer (1902*) described a chronic skin disease called acrodermatitis chronica atrophicans which was sometimes preceded by an ECM like lesion. A syndrome variously called tick-borne meningopolyneuritis, lymphocytic meningoradiculitis, chronic

* as quoted by Steere, (1989).

lymphocytic meningitis, and termed Bannwarth syndrome (Bannwarth 1944*). A few individuals with this syndrome developed erythema, radicular pain followed by chronic lymphocytic meningitis, and sometimes cranial or peripheral neuritis. Spirochete-like structures were observed in skin lesions of patients with Bannworth syndrome similar to ECM by Lenhoff (1948*). This finding resulted in the use of penicillin to treat such lesions in Europe, based on the assumption that this constellation of symptoms was due to a bacterial infection (Hollstrom, 1951*). In North America, an epidemic of juvenile rheumatoid arthritis in Lyme, Connecticut was found to be associated with ECM (Steere *et al.*, 1977). This clustering of the cases in three communities and follow-up studies concluded that Lyme disease was a multisystem illness that affected primarily the skin, nervous system, heart, and joints with a probable ixodid tick as vector for this malady (Steere *et al.*, 1978a; Steere *et al.*, 1978b).

Burgdorfer *et al.*, (1982) conclusively identified the spirochetal etiology of Lyme borreliosis transmitted by ixodid ticks of *Ixodes* spp. The spirochete was isolated by Barbour, (1984) and Johnson *et al.*, (1984a) described this organism as a new species of genus *Borrelia*, *Borrelia burgdorferi*. A large number of isolates of this organism have been recovered from a wide variety of hosts and geographical locations and they have been grouped under a single species. However, it has been shown that these strains are genomically and phenotypically heterogenous. Barnton *et al.*, (1992) studied 48 different isolates and divided them into three DNA groups (genospecies) based on patterns of specific rRNA gene restriction, analytical SDS-PAGE, and reactivity with murine monoclonal antibodies. Genospecies I contains 28 strains from Europe and the

United States and corresponds to *Borrelia burgdorferi* sensu stricto. Genospecies II contains 13 isolates from Europe and Japan and is named *Borrelia garinii* sp. nov. Genospecies III is termed as group VS461 and it includes 7 isolates from Europe and Japan. This heterogeneity has far reaching implications on the clinical picture, pathogenesis, host responses to Lyme borreliosis and vaccine development.

Vector: The vector species include *I. scapularis* in northeastern and Midwestern United States (Steere and Malawista, 1979), the western black-legged tick *I. pacificus* in the western United States (Burgdorfer *et al.*, 1985), *I. ricinus* in Europe (Krampitz, 1986), and *I. persulcatus* in Asia (Dekonenko *et al.*, 1988). Conspecificity of *I. dammini* and *I. scapularis* has since been established (Oliver *et al.*, 1993). In this literature review, both *I. dammini* and *I. scapularis* have been used based upon the original author's usage in the particular study being discussed.

In the United States, the preferred host of larvae of *I. scapularis* is the white-footed mouse, *Peromyscus leucopus*, which acts as the reservoir host for the spirochetes (Levine *et al.*, 1985). Larval ticks feeding on infected mice during late summer acquire spirochetes that are maintained through the larval molt and resulting nymphs are also infected (transstadial transmission). These nymphs feed in late spring and early summer preferably on white-footed mice but can also readily attack humans. In the process, they infect mice previously unexposed to *B. burgdorferi* creating a source of infection for the next generation of larvae later in summer (Wilson and Spielman, 1985). The transmission of infection to humans is mostly through infected nymphs. In the unfed nymphs, spirochetes are mostly restricted to the midgut. When these nymphs attach to a new host and feed for several days, spirochetes replicate, penetrate through the midgut wall, invade

salivary glands and are transmitted to the tick's host via saliva (Ribeiro *et al.*, 1987; Schwan, 1996). An infected tick has to remain attached to the host for at least 48 hours to successfully transmit the spirochete (Piesman *et al.*, 1987, Piesman *et al.*, 1991). The adult ticks feed in fall on white-tailed deer which are important hosts for tick survival but are not a reservoir for the spirochete (Wilson *et al.*, 1988).

The preferred hosts of larvae and nymphs of *Ixodes pacificus*, vector of Lyme borreliosis in the western US, are lizards instead of mice (Lane and Lavoie, 1988). The lizards unlike mice are not susceptible to *B. burgdorferi* infection (Brown and Lane, 1992). In this region, an enzootic cycle is supported by dusky-footed woodrats (*Neotoma fuscipes*) and a nonhuman-biting tick, *Ixodes neotomae* (Brown and Lane, 1992). A few larvae and nymphs of *I. pacificus* that feed on infected woodrats instead of lizards are responsible for transmitting the infection to humans (Brown and Lane, 1992). *Borrelia burgdorferi* has been isolated from a wide variety of wild, laboratory, companion, and domestic animals such as rats, mice, hamsters, raccoons, dogs, cattle, horses, and birds (Barbour, 1988a).

Disease: Lyme borreliosis has emerged as a disease of global human and veterinary public health importance and it is the most common arthropod-borne infection of humans in the United States (Spach *et al.*, 1993). During 1992, Connecticut (53.6 cases per 100,000), Wisconsin (10.7) and California (0.8) reported the highest rates in the northeast, north central, and Pacific coastal regions, respectively. Rates in some counties in California, Connecticut, Massachusetts, New York and Wisconsin exceeded 200 cases per 100,000 (CDC, 1993).

Lyme borreliosis is a multisystem illness with a characteristic initial clinical symptom of an expanding circular skin lesion called erythema chronicum migrans (ECM) (Steere *et al.*, 1978a) which occurs in 50-70 % of patients (Sigal, 1992a). The rash is usually accompanied by symptoms such as fatigue, malaise, lethargy, headache, myalgia, arthralgia, and regional or generalized lymphadenopathy (Sigal, 1992a). The early form of disease can be treated successfully with the use of suitable antibiotics such as Doxycycline, 100 mg, orally, twice daily for 3-4 weeks in the initial stages of disease. However, failure to diagnose and treat the disease promptly can lead to a debilitating chronic form of Lyme disease which is characterized by involvement of the nervous system, heart, and development of destructive arthritis in joints (Steere *et al.*, 1987; Sigal, 1992a). This form of the disease requires much more aggressive antibiotic therapy e.g. Ceftriaxone, 2g daily or 1g twice daily, intravenous, for 2-3 weeks and may not respond favorably to the therapy (Sigal, 1992a). The clinical manifestations exhibited by patients with Lyme borreliosis differ in Europe and North America (Steere, 1989). Skin lesions such as acrodermatitis chronica atrophicans and neuroborreliosis are more prevalent in European patients with the chronic form of disease while arthritic manifestations are more common amongst North American patients (Steere, 1989). Although, the underlying cause of this variation is not clear but the genetic makeup of patients and heterogeneity of the causative agent may have a role.

Diagnosis: Several laboratory tests such as indirect fluorescent antibody (IFA) staining methods, enzyme linked immunosorbant assay (ELISA) and Western blotting combined with history and clinical symptoms of the patient have helped in the diagnosis of Lyme borreliosis (Magnarelli, 1995). For further confirmation, the spirochete has been

recovered from blood (Benach *et al.*, 1983), cerebrospinal fluid (Preac Mursic *et al.*, 1984), skin biopsies of ECM (Berger *et al.*, 1985), and synovial fluid (Schmidli *et al.*, 1988). This disease is, however, complicated by problems in the early diagnosis, resulting in false negative and more often false positive results (Steere *et al.*, 1993). ELISA, which detects IgM or IgG response to *B. burgdorferi*, is currently the preferred serologic assay and should be used for initial screening (Nocton and Steere, 1995). Positive results should further be subjected to confirmation with Western blotting to determine the reactivity to spirochete specific polypeptides such as 31 kDa OspA protein, 34 kDa OspB protein, and the 39 and 93 kDa polypeptides (Zoller *et al.*, 1991). The T cell proliferative assays have been used to diagnose Lyme borreliosis in seronegative patients but with inconsistent results (Dattwyler *et al.*, 1988). The PCR technique has been used to detect small quantities of *B. burgdorferi* DNA in various body fluids and tissue specimens, however, a major concern with this highly sensitive technique is the risk of contamination leading to false-positive results (Nocton and Steere, 1995).

Pathogenesis: The pathogenesis of a disease should be thoroughly understood before rational control measures can be developed. A rational vaccine construct will include the antigens inducing protective cell mediated immunity (CMI) and humoral immunity and would exclude other antigens which could possibly have some harmful effects. Such antigens need to be identified and characterized, however, it is important that the methods used imitate the natural disease process as faithfully as possible.

Persistence of the organism is one of the major elements in the pathogenesis of Lyme borreliosis. *Borrelia burgdorferi* causes chronic infections despite measurable B- and T-cell immune responses to spirochete immunogens (Garcia-Monco and Benach,

1989). The early IgM and IgG antibody response to the 41 kDa antigen of *B. burgdorferi*, expands to recognize an increasing number of spirochetal antigens with time. Sera from chronic Lyme borreliosis patients recognize an extensive repertoire of antigens by immunoblot (Craft *et al.*, 1986). Spirochetes can persist in the presence of high titers of *B. burgdorferi* specific antibodies. Similarly, detection of spirochetes in synovium (Johnston *et al.*, 1985) and skin (Asbrink *et al.*, 1984) of chronic patients by histopathology indicates the persistence of the organism.

An autoimmune component to the pathogenesis of Lyme borreliosis has been suggested. Cross reaction between *B. burgdorferi* and human neuronal antigens can occur (Aberer *et al.*, 1989; Sigal, 1992b). Cross-reactivity between *B. burgdorferi* flagellin and human axonal protein with a molecular weight of 64 kDa has been reported (Sigal, 1993). Dai *et al.*, (1993) showed that a monoclonal antibody (H9724) specific for the 41 kDa protein, flagellin, of *B. burgdorferi* cross reacts with a 64 kDa protein purified from the SK-N-SH human neuroblastoma cell line. This protein was identified to be a chaperonin-HSP-60. This can possibly result in autoimmune reaction leading to immune mediated damage to the host nervous system. Girourd *et al.*, (1993) found an association between the presence of auto reative antibodies against human heat shock protein 60 (huHsp60) and *B.burgdorferi* infection. Lewis rats immunized with a non-pathogenic strain of *B. burgdorferi* and a chloroform-methanol extract (nonprotein antigens) thereof produced antibodies that reacted with gangliosides asialo-GM1 and GM1 (Garcia-Monco *et al.*, 1993, 1995). Conversely, antibodies raised against asialo-GM1 and GM1 also cross-reacted with *B. burgdorferi* anigens. Results of these studies suggest that molecular mimicry may play a role in pathogenesis of Lyme disease.

The role of elaboration of various cytokines by host T- and B-cells in pathogenesis of Lyme borreliosis has been studied. Proliferative responses of T-cells to whole *B. burgdorferi* were observed in patients with chronic Lyme disease in the absence of detectable antibody levels. (Dattwyler *et al.*, 1991). However, authors noted that initial antibiotic therapy might have resulted in the lack of a humoral response and subsequent persistence of organisms in the central nervous system or other privileged sites might have resulted in the chronic form of the disease. Patients with prolonged episodes of Lyme arthritis have T-cell responses directed against multiple spirochetal proteins (Yoshinari *et al.*, 1991). Peripheral blood lymphocytes obtained from such patients showed marked proliferative responses to 34, 41, 55/58, and 66 kDa polypeptides.

de Souza *et al.*, (1992) showed that naive splenocytes collected from both C3H/HeJ and BALB/c mice proliferated in response to *B. burgdorferi* spirochetes, as well as recombinant OspA and OspB. Proliferative responses were predominant among the B cell enriched fraction of splenocytes. The mitogenic effect was not similar to that induced by LPS since B-cells from C3H/HeJ mice (LPS-nonresponder) proliferated to the same degree as those from C3H/HeNCrlBr, which is an LPS responder strain.

Schoenfeld *et al.*, (1992) showed that sonicated preparations of washed spirochetes have potent mitogenic activity for B-lymphocytes from naive C57BL/6, C3H/HeJ, or BALB/c mice. This activity was only slightly inhibited by polymyxin B. This indicates that proliferation was not due to an endotoxin like molecule since such proliferation is inhibited by polymyxin B. B-cells not only proliferated but also differentiated into antibody secreting cells. In the same study, *B. burgdorferi* stimulated the release of IL-6 from splenocyte cultures. Polyclonal activation of B-cells, along with

production of pro-inflammatory cytokine IL-6, could ultimately lead to the appearance of autoreactive antibodies which could be involved in the pathogenesis of Lyme borreliosis (Schoenfeld *et al.*, 1992). Proliferative responses were not inhibited by polymyxin B indicating that a lipid A-containing lipopolysaccharide (LPS) was not involved in these responses.

In a follow up study, Tai *et al.*, (1994) demonstrated that normal human B-lymphocytes, but not T-lymphocytes, proliferated when incubated with either sonicated *B. burgdorferi* or purified OspA. Production of a high level of IL-6 by mononuclear cells was observed in response to stimulation by both sonicated *B. burgdorferi* and purified OspA (Tai *et al.*, 1994).

Lim *et al.*, (1994), established that severe destructive arthritis can develop in hamsters vaccinated with a whole-cell preparation of formalin-inactivated *B. burgdorferi* sensu stricto isolate C-1-11 in adjuvant, and followed with a challenge of a homologous strain before high levels of protective borreliacidal antibodies developed. Once high levels of protective antibody were produced, hamsters were protected from homologous challenge and subsequent development of arthritis. However, vaccinated hamsters still developed severe destructive arthritis when challenged with other isolates of three genomic groups of *B. burgdorferi* sensu lato (*B. burgdorferi* sensu stricto isolate 297, *B. garnii* isolate LV4, and *B. afzelli* isolate BV1). Humorally mediated responses did not seem to be responsible for the development of arthritis since passive transfer of serum from hamsters vaccinated with the same whole-cell preparation did not induce arthritis when naive syngeneic recipient hamsters were challenged with the homologous isolate or other isolates of *B. burgdorferi* sensu lato, even after daily administration of serum for 7

days (Lim *et al.*, 1994). Involvement of a cell mediated response was hypothesized for arthritis development.

In a follow-up study, T-lymphocytes obtained from inbred LSH hamsters vaccinated with the same whole-cell preparation as described above, conferred on naive recipient hamsters the ability to develop severe destructive arthritis when, challenged with either homologous or heterologous strains of *B. burgdorferi* (Lim *et al.*, 1995a). *Borrelia burgdorferi*-specific T-lymphocytes could not confer protection on hamsters receiving the adoptive transfer against infection with isolates of *B. burgdorferi* sensu stricto, since spirochetes were readily recovered from their tissues. This indicates that T-lymphocytes are not involved in the development of cell mediated resistance but contribute to development of severe destructive arthritis. $CD4^+$ T-lymphocytes are involved in the development of severe destructive Lyme arthritis (Lim *et al.* 1995b). When hamsters vaccinated with a whole-cell preparation of Formalin-inactivated *B. burgdorferi* organisms adjuvant were depleted of $CD4^+$ T lymphocytes and challenged, they failed to develop severe destructive arthritis. If the repopulation of vaccinated hamsters with $CD4^+$ T-lymphocytes was allowed by decreasing the number of anti-CD4 treatments, severe destructive arthritis readily occurred. Although, the cytokine profile of the cells responsible for development of severe destructive arthritis was not determined, authors suggested that these might be Th1-lymphocytes.

Cytokines play a very significant role in regulating the initiation and maintenance of immune responses against infectious diseases (Fresno *et al.*, 1997) The role of proinflammatory cytokines such as interleukin-1 (IL-1) and tumor necrosis factor-α

(TNF-α) in the pathogenesis of Lyme borreliosis has been studied. Defosse and Johnson, (1992) reported elevated levels of TNF-α in the sera and synovial fluids from patients seropositive for *B. burgdorferi*. Interleukin-1 is a potent mediator of inflammation (Dinarello, 1988a and b). Habicht *et al.*, (1985) showed that *B. burgdorferi* stimulates the release of IL-1 activity from human peripheral blood monocytes and a murine macrophage cell line. Kenefic *et al.*, (1992) reported the ability of high- and low-passage isolates of *B. burgdorferi* to stimulate the release of IL-1 activity from bovine peripheral blood monocytes and that it might contribute to the pathogenesis of arthritis.

Thioglycollate elicited peritoneal macrophages obtained from BALB/c mice produced elevated levels of IL-1 and TNF-α when incubated with *B. burgdorferi* strain 297 (Ramachandra *et al.*, 1993). This effect was not due to the presence of LPS since addition of polymyxin-B sulfate did not inhibit the elaboration of these cytokines. In contrast, macrophages from C3H/HeJ mice failed to produce IL-1 levels significantly higher than control and no TNF-α activity was noted in the culture supernatants. This study showed that these cytokines and their relative amounts are important in the pathogenesis of Lyme borreliosis. C3H/HeJ mice suffer from moderate to severe disease (Barthold *et al.*, 1990), whereas BALB/c suffer from a milder form of disease resembling Lyme borreliosis of humans (Barthold *et al.*, 1990). This difference in severity of disease might be related to the cytokine profile during the infection. Interleukin-6 is also a proinflammatory cytokine (Hirano *et al.*, 1990). *Borrelia burgdorferi* induced a dose dependent increase in IL-6 production by C6 rat glioma cells (Habicht *et al.*, 1991). This

production of cytokine by CNS cells might have a role to play in the pathogenesis of neuroborreliosis.

Studies mentioned above indicate that several mechanisms might be responsible for the final outcome and pathogenesis of Lyme borreliosis. Direct invasion and persistence of spirochetes in host tissue combined with cross-reactivity of spirochetal antigens with host tissue (auto-immune mechanism) is a major factor. Mitogenic activity of spirochetal antigens for host B-lymphocytes, antigen-specific proliferation of host T-cells to *B. burgdorferi* antigens and elaboration of pro-inflammatory cytokines like IL-1, IL-6, and TNF-α are the other contributing factors in Lyme disease pathogenesis.

Vaccine Development Studies: A suitable vaccine for prophylaxis of Lyme borreliosis is desirable. Immunization with whole live and killed spirochetes has been shown to induce protective antibodies capable of killing the spirochetes *in vitro* or preventing disease/infection in a number of animal models (Chu *et* al., 1992; Hughes *et* al., 1993; Jobe *et* al., 1994; Johnson *et al.*, 1986a; Johnson *et al.*, 1986b; Johnson *et al.*, 1988; Levy *et al.*, 1993; Lovrich *et al.*, 1991; Pavia *et al.*, 1991; Schaible *et al.*, 1993; Schmitz *et al.*, 1990; Schmitz *et al.*, 1991).

Hamsters vaccinated with whole-cell preparation of formalin-inactivated *B. burgdorferi*, develop severe destructive arthritis, when challenged with the homologous strain of the spirochete prior to development of protective levels of borreliacidal antibody (Lim *et al.*, 1994). A possible autoimmune reaction following immunization with whole spirochete suggests that a whole cell vaccine construct may not be a practical

prophylactic measure. An alternative approach is development of a subunit vaccine that consists of immunoprotective antigens of *B. burgdorferi*.

At least 30 different *B. burgdorferi* proteins have been identified (Craft *et al.*, 1986). Several outer surface proteins (Osp's) such as OspA (31 kDa), B (34 kDa), C (22 kDa), D (flagellin, 41 kDa), E (19 kDa), and F (29 kDa) have been screened as vaccine candidate antigens in addition to a 39 kDa protein (Seiler and Weis, 1996).

Fikrig *et al.*, (1990) demonstrated that mice immunized with recombinant OspA were protected against *B. burgdorferi* infection. Similarly, Simon *et al.*, (1991a) reported complete protection of severe combined immunodeficient (SCID) mice against a virulent challenge with *B. burgdorferi* following passive transfer of monospecific, hyperimmune anti-OspA (recombinant or native) serum. Monoclonal antibodies to flagellin (41 kDa), p20, p65, and p70 did not protect the mice (Fikrig *et al.*, 1990). Vaccination with only full length recombinant OspA protected mice against Lyme borreliosis whereas overlapping truncated fragments failed to do so (Bockenstedt *et al.*, 1993).

Subsequently, OspA has been evaluated extensively as a vaccine candidate antigen (Fikrig *et al.*, 1992a; Fikrig *et al.*, 1992b; Keller *et al.*, 1994; Sadziene *et al.*, 1993a; Sambri *et al.*, 1993; Schaible *et al.*, 1990; Schaible *et al.*, 1993; Simon *et al.*, 1991a; Simon *et al.*, 1991b; Stover *et al.*, 1993; Telford *et al.*, 1993). Anti-OspA antibody mediates its protective effects by destroying the spirochetes within the tick gut (de Silva *et al.*, 1996). This observation signifies the necessity of existence of high titers of anti-OspA antibodies at the time of exposure to a Lyme borreliosis infected tick.

OspA has emerged as the most promising vaccine candidate antigen and first clinical trials to examine safety and immunogenicity of a recombinant OspA vaccine in

humans were performed (Keller *et al.*, 1994). However, there are some serious concerns regarding the use of an OspA based Lyme borreliosis vaccine. A possible association of arthritis in human Lyme borreliosis patients expressing human class II lymphocyte antigen (HLA) DR4 and DR2 serotype and anti-OspA and -OspB reactive antibodies were reported (Steere *et al.*, 1990 and Kalish *et al.*, 1993). These finding suggest that people with these HLA specificities might not be suitable candidates for vaccination with an OspA based vaccine. Moreover, heterogeneity in OspA amongst strains can result in lack of full cross protection to different strains of *B. burgdorferi* (Barbour *et* al., 1985). Other studies have also indicated concerns regarding heterogeneity and lack of cross protection amongst different strains of *B. burgdorferi* (Barbour, 1988b; Fikrig *et* al., 1992c; Hovind-Hougen *et* al., 1986; Jonsson *et* al., 1992; LeFebvre *et* al., 1989; Wilske *et* al., 1986; Wilske *et* al., 1988; Wilske *et* al., 1993; Zumstein *et* al., 1992). Vaccination studies supported *in vitro* findings, confirming that vaccination with a single OspA type does not provide complete protection against challenge with diverse *B. burgdorferi* isolates (Lovrich *et al.*, 1995). Antisera generated by immunization with recombinant OspA from *B. burgdorferi* sensu stricto S-1-10 and C-1-11, *B. afzelli* BV-1, and *B. garnii* were unable to kill spirochetes from heterologous strains, but the homologous strain was killed in an *in vitro* assay. Loss of OspA expression on the spirochete within the tick before transmission to the host can potentially render the anti-OspA antibodies ineffective.

Borreliacidal properties of anti-OspB antibodies have been reported (Coleman *et al.*, 1992). Sadziene *et al.*, (1993b) selected a mutant with reduced expression of truncated OspB protein which had reduced (only 37 % of wild type) penetration into

human endothelium umbilical vein cell monolayer and required 30-300 fold higher numbers of spirochetes to establish infection in *SCID* mice by intradermal route of infection. This observation suggests a role for OspB in establishment of infection and possibly in pathogenesis. OspB has not received nearly as much attention as a vaccine immunogen as OspA. However, it has been the topic of some of the studies in the literature (Fikrig *et* al., 1992b; Probert and LeFebvre, 1994; Sadziene *et al.*, 1993a; Sambri *et al.*, 1993; Telford *et al.*, 1993).

Protective immunity can be induced in hamsters by immunization with a *B. burgdorferi* mutant that lacks OspA and OspB (Hughes *et al.*, 1993). Clearly, other potential vaccine candidate antigens exist. OspC has been the focus of attention because of the observation that expression of this molecule is up regulated on the spirochete surface in the feeding tick with a concurrent down regulation of OspA (Schwan *et al.*, 1995). *Ixodes scapularis* larvae were allowed to become infected with *B. burgdorferi* by feeding on infected mice. Following molting, 100 % of the unfed nymphs were found to be abundantly positive for expression of OspA by indirect immunofluoresence but none was positive for OspC. However, spirochetes in the midgut of partially fed nymphs became positive for the expression of OspC but the expression of OspA was decreased (Schwan *et al.*, 1995). Thus, anti-OspA antibody will be effective only in a very narrow window of time before the OspA expression is lost before or during the transmission of the spirochete (de Silva *et al.*, 1996). Immunization of outbred mice with recombinant OspC protected them against tick transmitted infection of *B. burgdorferi* (Gilmore *et al.*, 1996). OspC purified by preparative SDS-PAGE although immunogenic, did not elicit protection, suggesting the presence of a protective conformational epitope. Unlike OspA,

anti-OspC antibody does not cause destruction of spirochetes within the tick gut. There are other reports regarding the use of OspC as a vaccine antigen (Preac-Mursic *et al.*, 1992; Probert and LeFebvre, 1994).

C3H/HeJ mice immunized with recombinant OspF were partially protected from both intradermal needle inoculation and tick-mediated transmission of *B. burgdorferi* (Nguyen *et al.*, 1994). However, vaccination with recombinant OspE did not provide any protection (Nguyen *et al.*, 1994). A 75 and 90 % reduction in the spirochete load was detected in infected nymphal ticks following feeding on OspE and OspF immunized mice, respectively. Since it takes at least 48 hours following tick attachment for the transmission of spirochete (Piesman *et al.*, 1987), killing of spirochetes within the tick could have resulted in a smaller spirochete inoculum.

Scriba *et al.*, (1993) stimulated peripheral blood lymphocytes of seronegative donors *in vitro* with *B. burgdorferi* antigen. They prepared three human monoclonal antibodies (IgM isotype) which reacted with a 39 kDa spirochetal protein. These antibodies were borreliacidal in an *in vitro* assay. This protein was found to be the same that was described by Simpson *et* al., (1990) and has been named p39. An anti-p39 and not anti-OspA response was detected in inbred and outbred mice, following tick mediated transmission of the spirochete (Golde *et al.*, 1994). In addition, response to p39 did not seem to be MHC restricted, since all the strains of mice used in this study developed anti-p39 antibodies. These findings make p39 a suitable vaccine candidate for protection against Lyme borreliosis.

The Role of Vector Tick: Necessity of the tick vector adds another dimension to the discussion of the responses to *B. burgdorferi* pathogenesis and immune response to Lyme

disease. Effects of tick feeding on host hemostatic, inflammatory, and immune mechanisms have a very significant impact on disease transmission and establishment (Wikel, 1996b). Ticks being long term feeders must maintain a constant flow of blood in the bite site. Saliva of *I. dammini* contains the enzyme apyrase, which inhibits platelet aggregation by degrading adenosine diphosphate (ADP) (Ribeiro *et al.*, 1985). Prostaglandin E_2 in the saliva of *I. dammini* has potent vasodilatory effects and it is possibly anti-inflammatory by inhibiting neutrophil aggregation and mast cell degranulation (Ribeiro *et al.*, 1985). Saliva from *I. dammini* inhibited anaphylotoxin induced neutrophil aggregation, N-formyl-methionyl-leucyl-phenyl-allanine (FMLP) induced granule enzyme secretion, zymosan-induced superoxide secretion, and phagocytosis of *B. burgdorferi* spirochetes by up to 80 % (Ribeiro and Spielman, 1986). Anti-hemostatic effects of saliva from a variety of hematophagous ectoparasites including ticks were described by Champagne and Valenzuela (1996).

The anti-inflammatory effects of saliva are expressed by down regulation of various host pro-inflammatory mediators. *Ixodes dammini* saliva inhibited anaphylotoxin activity (Ribeiro and Spielman, 1986) and complement activity (Ribeiro, 1987). *Dermacentor andersoni* salivary gland extracts downregulated the elaboration of macrophage pro-inflammatory mediator interleukin-1 (IL-1) by 89.8 % and that of tumor necrosis factor-α (TNF-α) by up to 94.6 % (Ramachandra and Wikel, 1992). In addition, IL-2 and interferon-γ (IFN-γ) levels were also suppressed. The role of these cytokines in initiation, maturation and regulation of host immune response is well documented (Dinarello, 1988a and b; Perussia *et al.*, 1988).

Immunosuppressive properties of hematophagous, ectoparasitic arthropod saliva have been described by Wikel (1996a and b). Ticks are capable of reducing the proliferative responses of different vertebrate host lymphocytes to various mitogens (Wikel, 1982; Schorderet and Brossard, 1994, Ramachandra and Wikel, 1992). Inhibition of *in vitro* lymphoproliferative responses to phytohemagglutinin (PHA) was attributed to PGE_2 in the saliva of *Boophilus microplus* (Inokuma *et al.*, 1994). A protein with molecular weight of 5 kDa or greater in *I. scapularis* saliva inhibited the proliferation of mouse splenocytes to Concanavalin A (Con A), and PHA (Urioste *et al.*, 1994). One or more polypeptides/proteins in the molecular weight range of 36-43 kDa, obtained by preparative SDS-PAGE of *D. andersoni* salivary gland suppressed the proliferative responsiveness of murine splenocytes to Con A *in vitro* (Bergman *et al.*, 1995).

Since the spirochete is transmitted to the host at approximately 48 hours following tick attachment (Piesman *et al.* 1987; Ribeiro *et al.*, 1987), it can be postulated that by that time there will be a significant level of immunosuppression at the bite site. This makes the bite site immunocompromised in that a variety of host inflammatory and immune mechanisms are downregulated. Thus, making the local microenvironment more favorable site for spirochete establishment.

Recently, studies using two separate approaches demonstrated the role of tick in transmission of the spirochetes. Zeidner *et al.*, (1996) hypothesized that reconstitution of cytokines that are suppressed as a result of tick feeding may alter the dynamics of tick feeding and disease transmission. They administered cytokines such as TNF-α, IL-2, and IFN-γ to C3H/HeJ mice for 10 days during an infestation with *B. burgdorferi* infected *I.*

scapularis nymphs. Up to 95 % protection was seen in mice receiving TNF-α. Fifty five to 70 % of mice administered IFN-γ or IL-2, resisted acute infection as compared to untreated controls, in which the vector induced infection rate was 83.3 %.

Wikel *et al.*, (1997) infested female BALB/c mice four times with pathogen-free *I. scapularis* before a final infestation with *B. burgdorferi* infected nymphs. Although, no sign of acquired resistance to ticks was observed, repeatedly infested mice became resistant to tick transmitted Lyme borreliosis. Only 16.7 % of repeatedly infested mice became infected as compared to 100 % of non-infested controls when challenged with infected nymphs. Repeated infestation might have enabled the host to counteract the immunosuppressive and anti-inflammatory effects of tick feeding. This could have resulted in inhibition of transmission of spirochete because of changed microenvironment at the bite site which may not be conducive to establishment of infection.

Surface Antigen Modulation by the Spirochete: *Borrelia burgdorferi*, by virtue of being a tick-borne pathogen has to adapt to two very different microenvironmental and metabolic parameters i.e. ixodid tick, a poikilothermal arthropod and a homeothermal mammalian host. It is likely that to survive in these vastly different conditions, spirochetes undergo significant structural and metabolic changes when transmitted from the tick to the host. In fact, studies have demonstrated that growth temperature changes can lead to protein profile change and there are proteins that are only expressed in the mammalian host and others that are expressed at a higher level within the tick.

Cluss and Boothby (1990) studied changes in the protein profiles of *B. burgdorferi* following shifts from 28 °C to higher growth temperatures *in vitro*. An

increase in synthesis of four proteins designated heat stress protein (HSP) 1 through 4 (75, 42, 39, and 27 kDa, respectively) was noted. In contrast, the amount of a 29.5 kDa protein called heat labile protein was decreased at higher temperatures. In a similar study, Stevenson *et al.*, (1995) noted an increase in OspC expression levels in the lysates of B31 and N40 strain of *B. burgdorferi,* when growth temperature was shifted from 23 to 35 °C. Additional antigens with apparent molecular weights of 16, 19, 37, 38, 45, and 52 kDa were also present at a higher level in cultures of B31 strain grown at 35 °C than those grown at 23 °C. In the case of N40, four antigens, in addition to OspC, with apparent molecular weights of 18, 20, 37, and 45 kDa, were present in the 35° C lysate at higher levels than in 23 °C lysate.

Champion *et al.*, (1994) reported the cloning, sequencing and molecular analysis of the gene *eppA* (exported plasmid protein A) from virulent *B. burgdorferi* B31, which encodes a protein with calculated molecular weight of 17,972 D. Sera from patients with Lyme disease and rabbits experimentally infected with homologous spirochete recognized recombinant *eppA* (r*EppA).* However, hyperimmune rabbit antiserum to r*EppA* was unable to detect the presence of this protein in extracts of spirochetes cultured in BSK II or in the culture supernatants. This protein is an outer membrane or secreted protein which is only expressed *in vivo*. Similarly, Suk *et al.*, (1995) identified a novel protein, p21 which is expressed only in spirochetes infecting mice but not on culture grown borreliae. Wallich *et al.*, (1995) identified a gene pG which was expressed only during infection *in vivo*. Akins *et al.*, (1995) described an OspF homologue which is expressed by *B. burgdorferi* only in infected animals but not in cultured organisms.

Montgomery *et al.*, (1996) used fluorescent antibody to monitor the expression of OspA and OspC during the course of Lyme borreliosis in mice. The spirochetes recovered by peritoneal lavage 30 days after the initiation of infection did not stain with anti-OspA antibody but did so with anti-OspC antibody. However, the original inoculum expressed only OspA.

Schwan *et al.*, (1995) showed that the spirochetes in unfed *I. scapularis* nymphs express large amounts of OspA but no OspC, however, in ticks fed to repletion on mice, the converse was true. This switch in part was regulated by temperature change in that OspC was produced at 32-37 °C but not at 24 °C. Blood feeding was noted to be the other trigger. Passive administration of anti-OspA antibody can protect mice only if it is administered 24 hours prior to or at the time of attachment of infected ticks (de Silva *et al.*, 1996). Anti-OspA antibody provided protection by destroying the spirochetes within the tick gut. Thus, the presence of a high titer of anti-OspA antibody is a must in the brief window of time between tick attachment and spirochete transmission, since following the switch from OspA to OspC, anti-OspA antibody is ineffective. Natural infection in the case of individuals immunized with OspA based vaccines may not act as a booster because spirochete will have lost the OspA expression by the time they are transmitted to the host or shortly thereafter. Furthermore, host adapted borreliae that have no or low expression of OspA are unaffected by anti-OspA antibody and are not cleared from an OspA immunized individual (Barthold *et al.*, 1995), providing further proof of surface antigen modulation.

Scope and Significance of the Present Study: The immune response of the host to needle inoculated spirochete and to infection initiated by natural feeding of infected ticks

differs (Roehrig *et al.*, 1992). In most animals syringe inoculation of BSK culture-grown *B. burgdorferi* elicits a strong and rapid anti-OspA and -OspB antibody response (Benach *et al.*, 1988; Schmitz *et al.*, 1991). In contrast, no or late (months) anti-OspA and -OspB response is seen in humans and hamsters in tick transmitted disease (Craft *et al.*, 1986; Habicht, 1988). One hypothesis put forward to explain this difference is that *B. burgdorferi* occupies an immune-privileged site in the host (Habicht, 1988), but it seems unlikely because a rapid response to other spirochetal antigens is mounted (Roehrig *et al.*, 1992). Needle inoculation may deliver a large initial antigenic dose as compared to the bite of an infected tick. Hamsters infected by tick bite remain carriers for their life, but a large anti-OspA and anti-OspB response is not noticed (Roehrig *et* al., 1992). Ticks can play a significant role in altering the presentation of bacterial antigens to the host since complex interactions at the tick-host-pathogen interface and the effect of tick introduced immunogens on host immunoregulatory and effector pathways is critical to pathogen acquisition by the tick, transmission to the host, and establishment of tick-borne pathogen in the host (Wikel, 1996a and b). In most studies, the usual mode of challenge following active immunization of the host with either whole-cell preparations or with isolated antigens has been by intradermal or subcutaneous inoculation of BSK culture grown *B. burgdorferi*. These routes of inoculation and the spirochetal source do not accurately reflect natural infection since it does not take into account the role of the tick during the transmission of the spirochete (Roehrig *et al.*, 1992).

Above outlined differences in the humoral response to needle inoculated and tick transmitted infections of Lyme borreliosis are known and more information regarding the modulation of spirochete surface antigens in relation to the tick are becoming known.

However little information regarding the temporal development of differential immune responses (both cellular and humoral), isotypes of the antibodies involved, antigens and their role in protective immune responses is available. We proposed to study the temporal development of these differences by collecting the sera from BALB/c mice at regular time intervals starting at three days post-infection through 16 weeks post-infection following needle inoculated or tick transmitted infection. At all intervals, sera were tested for *Borrelia*-specific antibodies, their titer, isotype, and the antigens they recognize in the whole *Borrelia* profile. We used ELISA (isotype specific) and immunoblotting to achieve this objective. The role of antibodies in protective immune response was assessed by an *in vitro* spirochete neutralization test.

This study endeavored to identify the differences in cell mediated immune responses of BALB/c mice to *B. burgdorferi* infection initiated by needle inoculation of culture grown spirochetes and those to an infection established by infestation of infected *I. scapularis* nymphs. Although a relatively better understanding of humoral immune responses to *B. burgdorferi* is available, not much is known about the role of cell mediated immune responses. We collected splenocytes from mice infected by either method at similar time intervals as for the studies on humoral immune responses and studied their proliferative responses to whole *B. burgdorferi* sonicate. Further, we fractionated the whole-cell sonicates by preparative SDS-PAGE into discreet molecular weight fractions. The proliferative responses of splenocytes collected from mice infected by needle inoculation or tick transmission were monitored in response to these fractions.

CHAPTER II

MATERIALS AND METHODS

Experiments were conducted to study the humoral and cellular immune responses of BALB/c mice to *B. burgdorferi* by using either needle inoculation of culture grown spirochetes or by infestation with infected *I. scapularis* nymphs. BALB/c mice were infected by either method and systematic studies of sera and splenocytes collected from these mice, at different time intervals post-infection, were conducted. Humoral responses to *B. burgdorferi* infection were evaluated in terms of time of appearance of *Borrelia*-specific antibodies, their isotype, titer, specificity, and *in vitro* spirochete neutralization ability. Differences in these parameters between the sera collected from mice infected by needle inoculation versus tick transmitted infection were determined. Groups of mice injected with sterile phosphate buffered saline (PBS, pH 7.2, 0.15 M, Appendix I, Section 1) or infested with pathogen free *I. scapularis* nymphs served as controls for needle inoculated and infected tick infestation initiated infection, respectively.

Systematic studies to determine the cellular responses of BALB/c mice to Lyme borreliosis were conducted. Mice infected by either needle inoculation of spirochete or by infestation with spirochete infected *I. scapularis* nymphs were sacrificed at selected time intervals following initiation of infection to collect splenocytes. Their *in vitro* proliferative responses to whole spirochete extract and isolated borrelial antigens obtained by preparative scale SDS-PAGE were determined. Once again, mice injected with sterile PBS or infested with pathogen free *I. scapularis* nymphs served as controls for needle inoculated and infected tick infestation initiated infection, respectively.

Organism and Culture Conditions

The *B. burgdorferi* isolate designated B31 was used in this study. It was isolated in pure culture from the midgut of an *I. scapularis* tick collected on Shelter Island, NY (Burgdorfer *et al.*, 1982). A low passage (Passage 2) culture of *B. burgdorferi* type strain B31, was obtained from the Centers for Disease Control (CDC), Fort Collins, Colorado. This organism was cultured in Barbour-Stoenner-Kelly II (BSK II, Appendix I, Section 1) medium at 34° C in a bacteriological incubator with air atmosphere (Barbour, 1984). *Borrelia burgdorferi* no longer expresses certain antigens following repeated passages in culture (Ramachandra *et al.*, 1994). In order to insure the use of a uniform population of spirochetes and the uniformity of *B. burgdorferi* antigenic profile during this study, a master seed of this low passage organism was prepared and it was maintained frozen in 10% bovine serum albumin fraction V (BSA) at -80°C. A cryovial containing two ml of *B. burgdorferi* culture (passage 2) was rapidly thawed by holding in a 37 °C water bath and one ml of this culture was inoculated in nine ml of BSK II in a 15 ml screw cap cell culture tube (Corning, New York, NY). After an incubation of 24 hrs at 34°C in a dry bacteriological incubator, culture was mixed by gentle pipetting and wet smear was prepared by placing 10 µl of culture on a microscopic slide and covering it with a cover slip. The viability and activity of spirochetes in this unstained smear was determined by dark field microscopy at a magnification of 200 X. Five ml of very motile spirochete culture was inoculated in 35 ml of BSK II medium (four tubes total) in a 50 ml conical tissue culture tube (Falcon, Franklin Lanes, NJ). Following a further incubation of 48 hrs at 34 °C, the spirochetes were centrifuged at 2000 X g for 30 minutes at 10 °C using

aerosol protection. Pellets obtained from all the four tubes were resuspended in 50 ml of fresh BSK II medium and mixed with an equal volume of 20 % BSA, in PBS. Two ml aliquots of this suspension were stored frozen at -80 ^{o}C in an ultra low temperature freezer (Environmental Equipment Company, Cincinnati, OH). For individual experiment, a vial of frozen culture was rapidly thawed by holding it in a 37 ^{o}C water bath and inoculated in fresh BSK II medium. A Petroff-Hauser counting chamber (Hausser Scientific Company, Horsham, PA) and dark field microscopy was used to enumerate the spirochetes. A cover slip was placed on the ruled area of the chamber and it was charged with 1: 100 diluted culture suspension. The number of spirochetes per ml were determined by the formula: number of spirochetes counted in the central square mm area of the chamber X dilution factor X 50,000. In addition to the type strain B31, a mutant of the same strain of B31 was procured as a generous gift from Dr. A.G. Barbour, Department of Microbiology, University of Texas Health Center, San Antonio, TX. This mutant lacks the 49 kb operon coding for OspA (31 kDa) and OspB (34 kDa), and thus it does not express these two molecules. This strain was also expanded in BSK II and a master culture was stored in 10 % BSA at -80^{o} C as explained earlier

Experimental Animals

Female BALB/c mice, six to eight weeks old, weighing 20-25 g were used in this study. Mice were obtained from Jackson Laboratories, Bar Harbor, Maine and housed at Laboratory Animal Resources facility of School of Veterinary Medicine, Oklahoma State University, Stillwater, OK. at 22^{o} C, and were fed a commercial diet and water *ad libitum*.

Tick Colony

Adult, replete female *Ixodes scapularis* were collected from the fields around Stillwater, OK, placed in individual vials with plaster of Paris (POP) and charcoal at the bottom, and held in a desiccator maintained at 25° C for oviposition and subsequent hatching of eggs to obtain larvae. *Borrelia burgdorferi* is not transmitted by transovarial route, thus these larvae were pathogen free. Pathogen free nymphs were obtained by feeding these larvae on BALB/c mice. The bottom of a 1.5 ml conical microcentrifuge tube was removed and a hole was made in the lid for air circulation. Hair was trimmed from the back of a mouse and the tube was glued on it to create a site for infestation that could not be groomed. A total of 100-150 (not counted) unfed larvae were deposited into the capsule using a camel hair brush. A fine mesh cloth was used while closing the lid to allow air circulation and prevent the escape of larvae. Larvae were allowed to obtain a blood meal and capsules were checked every day for attachment and collection of replete larvae. Twenty five fed larvae were placed into individual vials containing POP plus charcoal at the bottom, held in a desiccator with water in the bottom and at 25° C until molting to the nymphal stage occurred.

In order to obtain *B. burgdorferi* infected nymphs, larvae were fed on BALB/c mice that were infected with *B. burgdorferi* strain B31. One vial (two ml) of frozen *B. burgdorferi* B31 master culture was rapidly thawed and one ml of culture was inoculated into each of two 15 ml screw cap tissue culture tubes containing nine ml of fresh BSK II. It was incubated at 34 °C for 24 hrs and viability and activity of the culture was checked by dark field microscopy as described earlier. One ml of this culture was reinoculated into nine ml of fresh BSK II and incubated further for 48 hrs at 34 °C in a bacteriological

incubator. At the end of this incubation period, the spirochetes were pelleted by centrifugation at 2000 X g for 30 minutes at 10 °C and washed twice by resuspending in sterile PBS and recentrifugation. Finally, spirochete pellets obtained from both tubes were resuspended in five ml of sterile PBS, they were counted using Petroff Hausser counting chamber and the final count was adjusted to 2.0 X 10^7 per ml. Each mouse was inoculated with 0.5 ml of this suspension (1.0 X 10^7 spirochetes/mouse) by intraperitoneal (IP) injection using a 22 gauze needle. Four weeks later, an ear punch biopsy was taken from each mouse to confirm infection by the method of Sinsky and Piesman, (1989). The ears of mice were surface sterilized by scrubbing with 70% ethyl alcohol and a small sterile punch was used to obtain a piece of tissue. It was placed in four ml of BSK II medium in a five ml screw cap tube without further triturition and incubated at 34 °C in a dry bacteriological incubator. After one week, a wet smear of culture medium was examined by dark field microscopy for the presence of spirochetes. Following confirmation of infection, mice were infested with unfed larvae and allowed to feed to repletion as described earlier. Replete larvae were maintained for molting into nymphs as described above.

Confirmation of Infection in *I. scapularis* Nymphs

Following molting, unfed nymphs were examined for spirochetal infection by dark field microscopy (Piesman *et al.*, 1987). Two unfed nymphs were randomly picked out of the nymphs from each of eight vials in which 25 fed larvae had earlier been placed for molting. They were placed in a 20 µl drop of PBS on a microscopic slide and finely minced with a sharp scalpel blade. A cover slip was placed on top to prepare a wet smear

that was examined by dark field microscopy at 200 X magnification. The unfed nymphs molted from larvae obtained following repletion on uninfected mice were also examined in a similar manner as control. Observation of one viable spirochete in the smear was considered positive infection of the tick.

Preparation of Whole Spirochete Extracts (WSE)

One vial (two ml) of frozen *B. burgdorferi* B31 master culture was rapidly thawed and one ml of culture was inoculated into each of two 15 ml screw cap tissue culture tubes containing nine ml of fresh BSK II. It was incubated at 34 °C for 24 hrs and viability and activity of the culture was checked by dark field microscopy as described earlier. Eight hundred ml of BSK II medium was distributed (40 ml per tube) in 20 sterile, 50 ml, conical, screw cap culture tubes (Falcon, Franklin Lakes, NJ) and inoculated with one ml of actively growing spirochete culture. Spirochetes were allowed to grow to late log phase, for approximately two weeks after inoculation. At this time the medium had turned yellow in color and large flakes of spirochetes could be seen settled at the bottom of tube. Little motility could be observed in spirochetes on examination of these cultures by dark field microscopy, although a large number of spirochetes were present. Spirochetes were killed by further addition of a stock solution of thimersol (10 mg/ml in distilled water, 400 µl/40 ml of culture suspension, 0.01% final concentration), and further incubating the cultures overnight. Spirochetes were pelleted by centrifugation at 2000 X g at 10 °C for 30 minutes. All the pellets (20) were pooled together. Spirochetes were washed three times with sterile PBS, to remove thimersol and media components, by repeated centrifugation and resuspension in sterile PBS. Finally,

spirochetes were resuspended in 10 ml of sterile PBS. Spirochetes were disrupted by sonication at 55,000 cycles/s of one minute in a water bath sonicator for a total of five minutes. WSE was held on ice between sonication cycles for cooling.

Protein concentration of the sonicate was determined by the microtiter plate bicinchoninic acid (BCA, Pierce, Rockford, IL) method of Smith *et al.*, 1985, using bovine serum albumin (2 mg/ml, in a 0.9 % aqueous NaCl solution, Pierce, Rockford, IL) as standard. The standard was diluted with PBS to a protein concentrations of 50, 100, and 150 μg per ml. Whole spirochete extract was serially diluted from 1 : 20 through 1: 640. Fifty parts of Solution A were mixed with one part of Solution B to obtain the working reagent for BCA protein assay. Two hundred and fifty μl of working reagent was deposited into each well of a flat bottom, 96 well plate (Corning, New York, NY). Twenty μl of each standard dilution (1, 2, and 3 μg total protein), and WSE dilution were added per well in triplicate. Twenty μl per well of PBS added in triplicate served as blank. The plate was covered with a lid and agitated on a Genie 2 vortex shaker (Scientific Industries, Bohemia, NY) for 2-3 minutes. Plate was incubated at 60 ^{o}C for 30 minutes and optical densities were determined at 562 nm using an automatic microplate reader (model no. EL307C, Biotek Instruments, Winsooki, VT). Absorbance values for each sample and standard were averaged and protein content of WSE was determined by linear regression. The final protein content of WSE was adjusted to one mg per ml. The WSE prepared from original B31 strain was used as antigen for determination of *B. burgdorferi* specific antibodies by ELISA, and for lymphocyte proliferation assay. The WSE was prepared in a similar manner from the OspA and B negative strain which also

served as the starting material for preparative SDS-PAGE fractionation (explained below) in addition to being used in the lymphocyte proliferation assay.

Fractionation of Whole Spirochete Extract

Initial attempts fractionate the WSE obtained from *B. burgdorferi* strain B31 were unsuccessful due to the presence of a large amount of OspA in this preparation. The entire spectrum of fractions obtained by preparative SDS-PAGE fractionation of this antigen using Prep Cell Model 491 (Bio-Rad Laboratories, Hercules, CA) was contaminated with OspA. Therefore, WSE prepared from the mutant strain of B31, which lacks the expression of OspA and B was used for this purpose.

Whole spirochete extract was fractionated using a Model 491 Prep Cell (Bio-Rad Laboratories, Hercules, CA). This apparatus can be used to separate specific proteins from complex mixtures by continuous elution electrophoresis. Proteins are electrophoresed vertically through a cylindrical polyacrylamide gel, which migrate off the gel as individual bands passing directly into an elution chamber that serves as a molecular sieve. Elution buffer is drawn radially inward to an elution tube and out to the fraction collector. Antigens are, therefore, eluted in different fractions according to their molecular weights.

Preparative SDS-PAGE fractionation was performed by the method outlined by Bergman *et* al.,(1995). All the buffers used and recipes for the preparation of gels with different acrylamide concentration are given in Appendix I (Section 2). Three separate fractionations were conducted using an approximately nine cm high resolving gel, with three different acrylamide concentrations, i.e. 7.5, 10, and 12 %. (Appendix I) and three cm stacking gel with 4 % acrylamide concentration (Appendix I) cast in 28 mm internal

diameter gel tube of the apparatus. Resolving gel was cast, over layered with distilled water and allowed to polymerize overnight. A variable speed pump (Bio-Rad Laboratories, Hercules, CA) circulated water at room temperature through cooling core of the gel tube to remove the heat produced during polymerization. In the morning, the top of the resolving buffer was rinsed with electrophoresis buffer and a three cm high stacking gel (4 % acrylamide) was cast and allowed to polymerize for one to two hours. The gel tube was assembled with upper and lower buffer chambers which were then filled with electrode buffer (Appendix I, Section 2).

Whole spirochete extract prepared from OspA and OspB negative mutant was prepared for discontinuous SDS-PAGE according to the method of Laemmli, (1970). Two ml of WSE (2 mg total protein) was mixed with 400 µl of 3 X sample buffer (Appendix I, Section 2) and heated to 95° C for five minutes. The sample was centrifuged at 14,000 X g to remove any suspended material. This sample was underlayed the electrode buffer in the upper chamber on top of the stacking gel using a syringe attached to fine tubing. The whole apparatus assembly was placed in a chromatography chamber maintained at 5 °C and connected to a variable speed Tris™ pump (Isco, Lincoln, Nebraska) and a fraction collector (Gilson Medical Electronics, Middleton, WI). Electrophoresis was conducted at 40 mA constant current using power supply model 1000/500 (Bio-Rad Laboratories, Hercules, CA). The fraction collector was started once the band of bromo phenol blue dye (present in the sample buffer) reached the bottom of resolving gel. Proteins eluting from the gel were captured in deaeriated electrode buffer

(elution buffer) flowing at the rate of one ml/min. A total of 128 fractions of 2.5 ml each were collected at 2.5 minute intervals for each prep-cell run.

Analytical SDS-PAGE of Fractions

Selected fractions over the entire spectrum of collected fractions were analyzed by analytical SDS-PAGE and gels were silver stained to determine the composition of each fraction (Morrissey, 1981). Analytical SDS-PAGE was conducted using Mini-PROTEAN II dual slab electrophoresis cell (Bio-Rad Laboratories, Hercules, CA). Four-screw clamp assemblies were used to prepare glass plate sandwiches using a pair of 0.75 mm thick spacers and aligned on the casting stand. A resolving gel with 12 % acrylamide concentration (Appendix I, Section 2) was poured and overlaid with water. It was allowed to polymerize for one to two hours. The top of the resolving gel was rinsed with electrode buffer and a four percent acrylamide stacking gel was poured on top of the resolving gel. A 10 well comb was placed in the stacking gel taking care not to trap any air bubbles. Stacking gel was allowed to polymerize for about one hour. Clamp assemblies along with gels were snapped onto the inner cooling core which also formed the upper buffer chamber. This whole assembly was placed in the lower buffer chamber and both buffer chambers were filled with electrode buffer.

Fifty µl of selected fraction was mixed with 10 µl of 3 X sample buffer and 25 µl of this sample was loaded per well. One of the wells was loaded with standard low molecular weight markers (Sigma, St. Louis, MO). Electrophoresis was carried out at 200 volts constant voltage for about 45 minutes or until the dye band reached the bottom of the resolving gel.

Gels were removed from the clamp assemblies following the completion of electrophoresis and stained with silver stain using a Silver Stain Plus kit (Bio-Rad Laboratories, Richmond, CA). Gels were fixed for 20 minutes in the fixative solution (100 ml methanol, 20 ml acetic acid, 70 ml water and 10 ml fixative enhancer concentrate). Gels were washed twice for 10 minutes with double distilled water and gentle agitation. Gels were stained with stain/developer solution (prepared by mixing 35 ml water, five ml silver complex solution, five ml reduction moderator solution, five ml image development reagent and 50 ml development acceleration reagent in that order with constant stirring). Gels were stained for about 20 minutes or until optimal band intensity was observed. Staining was stopped by draining the stain solution and adding a stop solution of five percent acetic acid in double distilled water.

Pooling and Removal of SDS From Fractions

After careful visual analysis of stained gels, the fractions with similar protein composition were pooled together. From the pools of fractions, excess SDS was removed by precipitation using a potassium salt (Suzuki and Terada, 1988). Dibasic potassium phosphate (K_2HPO_4, FW 174.18) was added to each fraction to a final concentration of 20 mM (50μl of 69.7 mg/ml solution in double distilled water per 950 μl of each fraction pool), incubated for 15 min. at room temperature and centrifuged for 15 minutes at 14,000 X g in a high speed centrifuge. Supernatants were collected, and dialyzed against PBS to remove tris. The constitution of each fraction pool was again determined by analytical SDS-PAGE (12 % resolving, 4 % stacking gel) as described for fractions. Subsequently, all the fraction pools were sterilized by passing through a 0.22 micron filter

(Millipore Corporation, Bedford, MA) and stored frozen in one ml aliquots at -20 °C until used in the *in vitro* lymphocyte proliferation assay.

Initiation of Infection in BALB/c Mice

Six to eight week old BALB/c mice were infected with *B. burgdorferi* isolate B31 by both needle inoculation of spirochetes grown in BSK II or by infestation with *B. burgdorferi* strain B31 infected *I. scapularis* nymphs. One group of mice (described below in experimental design section) was infected with needle inoculation using exactly same procedure as described for infection of mice for raising infected *I. scapularis* nymphs. Another group of mice was injected with 0.5 ml of PBS (IP) per mouse to serve as matched controls.

For initiation of tick transmitted infection, six *B. burgdorferi* B31 strain infected *I. scapularis* nymphs were infested per mouse in a similar fashion as the method explained for infestation of larvae. Each mouse was placed in an individual cage held over water to prevent the accidental escape of infected nymphs. A control group of mice was infested in a similar fashion with pathogen free nymphs. Mice were checked daily for attachment and feeding of nymphs. The nymphs were allowed to feed to repletion and the capsules were removed one week after the day of infestation.

Enzyme-linked Immunosorbant Assay (ELISA)

An ELISA developed and standardized by Ramachandra *et al.*, (1993) was used to monitor IgM and IgG immunoglobulins reactive with *B. burgdorferi* B31 WSE in the sera collected from different groups of mice at intervals explained in the experimental design section. Immulon-2, 96-well flat bottomed microtitration plates (Dynatech

Laboratories, Chantilly, VA) were coated with WSE (B31 type strain) at 50 µl/well (2 µg/ml in PBS, 0.15 M, pH 7.2, 100 ng total antigen/well) and plates were dried overnight in a dry bacteriological incubator at 34° C. The antigen containing wells were blocked with PBS-0.05% Tween 20 containing 5% horse serum, at 100 µl/well, for 1 h at 37 °C, and then washed three times with PBS-Tween 20. Next, 100 µl of test serum was added per well, serially diluted in the plate (1:20 to 1:20,480) and incubated for 1 h at 37 °C. Plates were washed again as above and 100 µl of horseradish peroxidase-conjugated rabbit anti-mouse IgG or IgM (Jackson Immunoresearch Laboratory, West Grove, PA) diluted to 1:5,000 in PBS-Tween 20 containing 1% bovine serum albumin was added to each well. Plates were further incubated for 1 h at 37° C. The plates were again washed three times and 100 µl of substrate solution containing 40 mg of o-phenylene diamine (Eastman Kodak, Rochester, NY), 30 µl of 30% hydrogen peroxide in 100 ml of phosphate citrate buffer (pH 5.0, Appendix I, Section 1) was added to each well and color allowed to develop for 15 minutes in the dark at 37 °C. Reaction was stopped by addition of 30 µl of 4N HCl per well and optical densities determined at 490 nm using a microplate reader (model no. EL307C, Biotek Instruments, Winsooki, VT).

Immunoblot Analysis of Sera

Immunoblot analysis of all the sera collected was carried out using Coomassie stained protein gels according to the method of Thompson and Larson (1992) as described by Ramachandra *et al.*, (1992). The SDS-PAGE of WSE (prepared from B31 strain of *B. burgdorferi*) was conducted using a Mini-PROTEAN II dual slab gel system (Bio-Rad, Richmond, CA) as described by Laemmli, (1970). Two hundred µl total

volume of WSE (type strain B31), containing 10 μg total protein was loaded in the trough formed in 4 % stacking gel and was resolved in a 12% acrylamide resolving gel. Standard Low Molecular Weight markers (Sigma, St. Louis, MO) were run along side.

Gels were stained with rapid Coomassie stain (Research Product International Corp., Mount Prospect, IL). Gels were fixed by incubating in 12.5 % trichloroacetic acid for 10 minutes with gentle agitation at room temperature. Working strength (1 X) stain solution was prepared by mixing two ml of stock stain solution with 40 ml of 7.5 % methanol-5 % acetic acid. Gels were stained for 20-40 minutes and then destained overnight in 7.5 % methanol-5 % acetic acid to remove background staining.

The stained proteins were electrotransferred to nitrocellulose membrane, using Mini Trans-Blot® electrophoretic transfer cell (Bio-Rad, Richmond, CA). Transferred proteins remained stained during the immunodetection and thus, were easier to locate and determine their molecular weight (Thompson and Larson, 1992). Coomassie stained gels were equilibrated in transfer buffer (Appendix I, Section 2) for 15 minutes to remove electrophoresis buffer salts and detergents. Nitrocellulose membrane (Bio-Rad, Richmond, CA) was cut to the size of the gel and soaked in transfer buffer by sliding it at an angle of 45° and allowed to soak for 15 minutes. Pre-cut filters and Fiber pads (Bio-Rad, Richmond, CA) were completely soaked in transfer buffer. Mini Trans-Blot electrode (Bio-Rad, Richmond, CA) was installed in the buffer chamber and buffer chamber was filled up to half with chilled transfer buffer. A one inch Teflon coated stir bar was placed at the bottom of buffer chamber. A Bio-Ice cooling unit (Bio-Rad, Richmond, CA) was prepared in advance, by filling it with deionized, distilled water and

storing in the freezer. The frozen cooling unit was installed in the buffer chamber, next to the electrode. The gel holder cassette was placed in a shallow glass dish containing cold transfer buffer with gray panel flat on the bottom of the vessel. A pre-soaked Fiber pad was placed on the gray panel. A piece of filter paper saturated with transfer buffer was placed on top of the Fiber pad. Surface of the filter paper was saturated with 2-3 ml of transfer buffer. Next, equilibrated gel was placed on top of the paper avoiding trapping of any air bubbles in between. Surface of the gel was flooded with transfer buffer and pre-wetted nitrocellulose membrane was placed on top of the gel. A small glass test tube was rolled over the top of the membrane (like a rolling pin) to eliminate any air bubbles trapped between the gel and the membrane. Surface of the membrane was flooded with transfer buffer and a sandwich was completed by placing a filter paper and a Fiber pad on top. Finally the cassette was closed by securing the latch. The gel holder cassette was placed in the buffer tank so that the gray panel of the holder faced the gray cathode electrode panel. The buffer tank was placed on top of a magnetic stirrer and filled with buffer to slightly above the level of the top row of circles on the gel holder cassette. The magnetic stirrer was turned on and the lid was put in place. Transfer of the proteins was performed at 100 V constant voltage for one hour.

The blots were removed from the gel holder cassette and stored in the dark until Western blot (immunoblot) analysis was carried out on them. Nitrocellulose blots were equilibrated in Tris-saline buffer (10 mM Tris-HCl, 0.9% NaCl, pH 7.4, Appendix I) for 15 minute and then incubated in Tris-saline buffer containing 5% non-fat dry milk (BLOTTO, Bovine Lacto Transfer Technique, Johnson *et al.*, 1983) for 45 minutes at room temperature to block non-specific protein binding sites.

Membranes were then transferred to a Mini-PROTEAN® II multiscreen apparatus (Bio-Rad, Richmond, CA), which enables screening of up to 20 different serum samples at the same time. Sera diluted to 1:30 in Tris-saline-BLOTTO were loaded into multiscreen channels (600 µl/channel) and incubated for 60 minutes at room temperature with gentle agitation. Serum samples were then removed by aspiration and membranes washed three times each with Tris-saline-BLOTTO and Tris-saline-BLOTTO-Tween 20 (BLOTTO containing 0.05% Tween-20) for five minutes with gentle agitation. Reacting antibodies were detected by HRPO-conjugated, affinity purified, goat anti-murine IgG (Jackson Immunoresearch Laboratories, Inc. West Grove, PA) used at a dilution of 1:200. Blots were incubated for one hour at room temperature. Immobilized labeled antibodies were detected by incubation with a substrate solution containing 60 ml of Tris -saline buffer, 30:1 of 30% hydrogen peroxide, 12 ml of 4-chloro-1-naphthol (3 mg/ml in anhydrous methanol) for 30 minutes at room temperature. Color development was stopped by removing the substrate and washing with Tris-saline buffer.

In vitro Spirochete Neutralization Assay

All antisera obtained at different times post-infection were tested for their ability to neutralize type strain B31 spirochetes *in vitro*. The method described by Lovrich *et al.* (1991) was followed with some major modifications. Spirochetes were grown to 4-6 X 10^7 /ml in BSK II after reviving a fresh vial of the master seed and adjusted to 2.0 X 10^6 /ml by adding fresh medium. 50µl (1.0 X 10^5 spirochetes) of this suspension were added in triplicate to 100 µl of 1:20 dilution of immune or control serum placed in a sterile 1.5 ml conical centrifuge tube. Twelve µl of guinea pig complement (at least 250

complement hemolytic units/ml, Gibco, Grand Island, NY) was added to the suspension and final volume brought to 200 µl by adding fresh BSK II medium. This mixture was incubated at 37° C in a humidified incubator with five percent CO_2 atmosphere (NAPCO, Precision Scientific, Chicago, IL) for 6 h. Then, 1 µCi of [^{3}H] adenine, specific activity 851 GBq/mmol, (20 µl in BSK II) was added along with 600 µl of fresh BSK II medium.

This mixture was further incubated for 96 h. At the end of the experiment, all spirochetes were inactivated with a one hour incubation at 37 °C in Thimersol (10 mg/ml in distilled water, 8.2 µl/tube, final concentration 0.1%). Amount of label incorporated was determined by immobilizing the nucleic acid content of each tube onto a glass fiber filter using an automated cell harvester (Brandel Laboratories, Gaithersburg, MD). Glass fiber filters were placed in six ml plastic, screw cap scintillation vials (Wheaton, VWR Scientific Products, Suwanee, GA) and covered with two ml Biodegradable counting Scinillant (Amersham, Arlington Heights, IL). The incorporation of methyl-tritiated adenine radioactivity on the filters was determined by counting in an automatic liquid scintillation spectrophotometer (Model LS6000SC, Beckman Instruments, Inc. Fullerton, CA). In some tubes heat inactivated (56 °C for 30 min.) pre-immune serum, with or without addition of complement was added instead of test serum to serve as control. Also, in some tubes only heat inactivated or normal complement alone (no serum) was added as controls for this assay.

In vitro Splenocyte Proliferation Assay

This assay was conducted as described by Ramachandra and Wikel (1992). Experimental or control subgroups of mice were sacrificed at different time intervals

post-infection as outlined in the experimental design section. One subgroup each from tick transmitted and needle inoculated infection groups was sacrificed providing four spleens per subgroup. Spleens from each subgroup were removed, pooled, and placed into sterile RPMI 1640 (Gibco, Grand Island, NY, Appendix I, Section 1) containing 10% heat inactivated (56 °C, 30 minutes) fetal calf serum (Gibco, Gaithersburg, MD), 2 mM glutamine, 100 units penicillin and 100μg streptomycin/ml. A single cell suspension was prepared in RPMI 1640 medium by mechanically disrupting the spleens between ground glass edges of sterile microscopic slides (Fisher Scientific, Pittsburgh, PA). Slides used for this purpose were precleaned by washing in running water for one hour, dried and autoclaved. Cells were washed three times by centrifuging at 600 X g for 15 minutes at 25 °C after resuspension in RPMI 1640 medium.

The cells were counted using a hemocytometer. Ninety μl of Turk's solution [0.01 g gentian violet (Mallinckrodt, Paris, KY), 3 ml glacial acetic acid, and 97 ml distilled water] were placed in each of three wells of a 96-well round bottom well plate (Becton Dickinson and Company, Lincoln Park, NJ) and 10 μl of cell suspension was serially transferred from each well to the next with thorough mixing making dilutions of 1:10, 1:100, and 1:1000. The hemocytometer was charged with appropriate dilution i.e. at which the cells could be easily counted. Cells were counted in two diagonally placed large squares and a mean was calculated. Cells per ml were calculated by the formula: Mean cell count X dilution factor X 10^4. Cell count was finally adjusted to 5.0 x 10^6 cells/ml.

One hundred μl ($5.0 X10^5$ cells) of this suspension was placed in each well of a 96-well plate. One hundred μl of medium containing one μg Con A (Calibiochem, La Jolla, CA), one μg *Escherichia coli* lipopolysaccharide (LPS) (Sigma, St. Louis, MO), one μg whole spirochete extract (*B. burgdorferi* type strain B31), one μg whole spirochete extract (*B. burgdorferi* OspA and B negative mutant) and different fraction pools obtained by preparative SDS-PAGE, diluted 1:2 in 2 X RPMI were added to the cells in triplicate. Cells without the addition of any mitogen or antigen served as negative control. Also, the splenocytes obtained from control groups were subjected to similar assay at the same time. The whole procedure was done in duplicate. In one instance, the plates were incubated for 54 h (3 day assay) and then pulsed with 1 μCi of tritiated thymidine (NEN Research Products, Boston, MA) per well (20 μl in RPMI 1640). In the second instance, cells were incubated for 102 h (5 day assay) before pulsing with tritiated thymidine. The amount of label incorporated was determined by immobilizing the nucleic acid content of each well onto a glass fiber filter using an automated cell harvester (Brandel Laboratories, Gaithersburg, MD). Glass fiber filters were placed in six ml plastic, screw cap scintillation vials (Wheaton, VWR Scientific Products, Suwanee, GA) and covered with two ml biodegradable counting Scinillant (Amersham, Arlington Heights, IL). The incorporation of methyl-tritiated thymidine radioactivity on the filters was determined by counting in an automatic liquid scintillation spectrophotometer (Model LS6000SC, Beckman Instruments, Inc. Fullerton, CA).

Experimental Design

The infected tick takes at least 36-48 hrs to transmit infection to the host following attachment (Ribeiro *et al.*, 1987). Therefore, to synchronize the initiation of *B. burgdorferi* infection by needle and tick transmission, the intraperitoneal injection of spirochetes in the group of mice receiving infection by needle inoculation was delayed until 3 days (72 hrs) after infestation with infected *I. scapularis* nymphs in the group of mice receiving tick transmitted infection.

Twenty mice were infested with infected *I. scapularis* nymphs (six nymphs per mouse). At the same time, 14 mice were infested with pathogen free *I. scapularis* nymphs (six per mouse) to serve as matched controls. The mice were checked daily and the nymphs were allowed to feed to repletion. Three days (72 hrs) later, twenty mice were injected with culture grown spirochetes (1.0×10^7 spirochetes/mouse), intraperitoneally. For this purpose, a vial of frozen master culture *B. burgdorferi* B31 strain was rapidly thawed and inoculated in nine ml of fresh BSK II medium and incubated at 34 °C. Twenty four hrs later, the spirochete activity in the culture was checked by dark field microscopy and one ml of this actively growing culture was again inoculated into nine ml of fresh BSK II medium in four replicates and incubated for 48 hrs at 34 °C. The cultures were once again checked for spirochete activity and viability by dark field microscopy. The spirochetes were washed three times with sterile PBS by centrifugation at 3000 X g at 10 °C for 30 minutes and resuspension. Aerosol protection was used at the time of centrifugation by placing the screw caps on the centrifuge tubes. The spirochetes were enumerated and the final count was adjusted to 2.0×10^7 spirochetes per ml. Each mouse

was injected with 500 µl (1.0 X 10^7 spirochetes) of this suspension by intraperitoneal route. Fourteen mice were injected with 500 µl of sterile PBS (IP) to serve as matched controls. The day of needle inoculation of spirochetes was deemed day zero for both methods of infection.

A pooled serum sample was collected by bleeding four randomly selected mice from each infected and control group on days three, 10 and 21. Four mice selected from each tick transmitted infection and needle inoculated infection groups were sacrificed at one, two, four, eight and twelve weeks post-infection. Blood samples were collected and pooled for the respective group to obtain a serum sample. Spleens were harvested and pooled to obtain splenocytes to study their proliferative responses to different mitogens and antigens. Finally, the urinary bladder from each mouse was dissected and placed in four ml of BSK II medium and incubated at 34° C to confirm infection. Four mice from each control group were also sacrificed at similar intervals and processed in the same manner with two exceptions. Control mice were not sacrificed at four weeks post-infection. At eight weeks post-infection, two mice instead of four, each from the groups injected with sterile PBS or infested with pathogen free nymphs were sacrificed to serve as the matched control.

Serum samples were collected at days three, seven, ten, 14, 21, 28, 56, and 84 post infection. The proliferative responses of splenocytes to different antigens and mitogens were studied at one, two, four, eight, and 12 weeks post infection.

CHAPTER III

RESULTS

Confirmation of Infection in *I. scapularis* Nymphs

A total of 16 nymphs (two nymphs from each of eight tubes containing 25 nymphs per tube) molted from larvae fed on *B. burgdorferi* infected BALB/c mice were examined. Observation of a single spirochete by dark field microscopy in a wet smear prepared from a triturated unfed nymph was considered to be confirmation of infection. The total number of spirochetes per smear was not counted. All the nymphs examined were found to be positive for infection. The unfed nymphs from these vials were used to infest BALB/c mice to establish tick transmitted *B. burgdorferi* infection. A same number of unfed nymphs received after molting from larvae fed on uninfected mice were also subjected to similar examination and spirochetes were not detected in any of these wet smears.

Tick Infestation

A total of 20, six to eight week old, female, BALB/c mice were infested with *I. scapularis* nymphs (six per mouse) confirmed to be infected with *B. burgdorferi*. Mice from this group were used to study different parameters of the immune response against tick-transmitted Lyme borreliosis. A total of 82 replete nymphs (68.33 %) were collected from 20 mice (120 unfed nymphs). 4.10 ± 0.19 (mean ± S.E) replete nymphs were obtained per mouse.

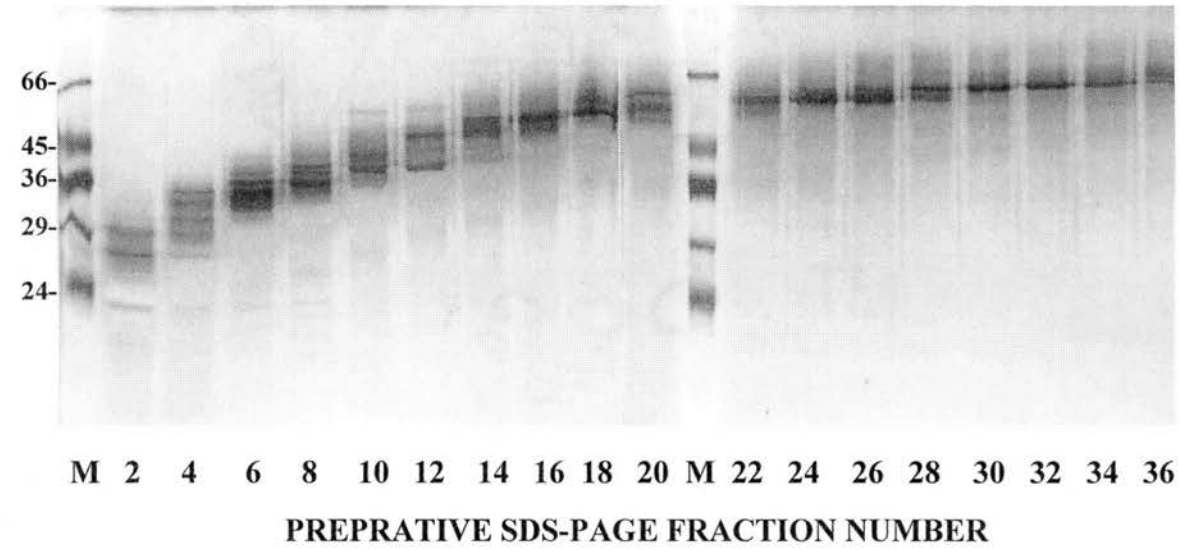
At the same time, fourteen mice were infested with pathogen-free *I. scapularis* nymphs (six per mouse) to serve as matched control for the group with tick-transmitted Lyme borreliosis. A total of 66 fed nymphs (78.57 %) were collected with a mean ± S.E. of 4.71± 0.32 per mouse. All the replete nymphs had dropped off from both the groups by day eight following infestation and the retaining capsules were removed from all the mice at that time.

Fractionation of Whole Spirochete Extract

Three different concentrations of resolving polyacrylamide gel (PAG) i.e. 7.5, 10, and 12 % were used in the 28 mm inner diameter column of the apparatus. The concentration of stacking gel was same for all the fractionations i.e. four percent. The heights of resolving and stacking gels were approximately nine cm and two cm, respectively. Two mg total protein of WSE (OspA and B deficient strain) in two ml volume was fractionated as described in the materials and methods section. A total of 128 fractions, each 2.5 ml in volume, were collected for each fractionation run. Selected fractions were analyzed by analytical SDS-PAGE to determine their composition.

Fractionation 1: Every other fraction, starting with 2nd fraction up to fraction 36 obtained by a preparative run using 7.5 % acrylamide concentration was examined for its protein composition using a 12 % analytical SDS-PAGE gel stained with silver (Figure 1). Fractions two, four, six, eight, 10, and 14 showed the presence of more than two bands in the molecular weight range of 29 kDa to 45 kDa. Fractions 16 and 18 showed a single major band at 45 kDa. Fraction 20 contained three bands in the molecular weight range of 45 to 51 kDa. Fractions 28, 30, 32, and 34 show one major band at about 56 kDa position.

Figure 1. Preparative scale, continuous-flow, SDS-PAGE fractionation of *Borrelia burgdorferi* (OspA and B deficient strain) whole spirochete extract using 7.5% acrylamide.



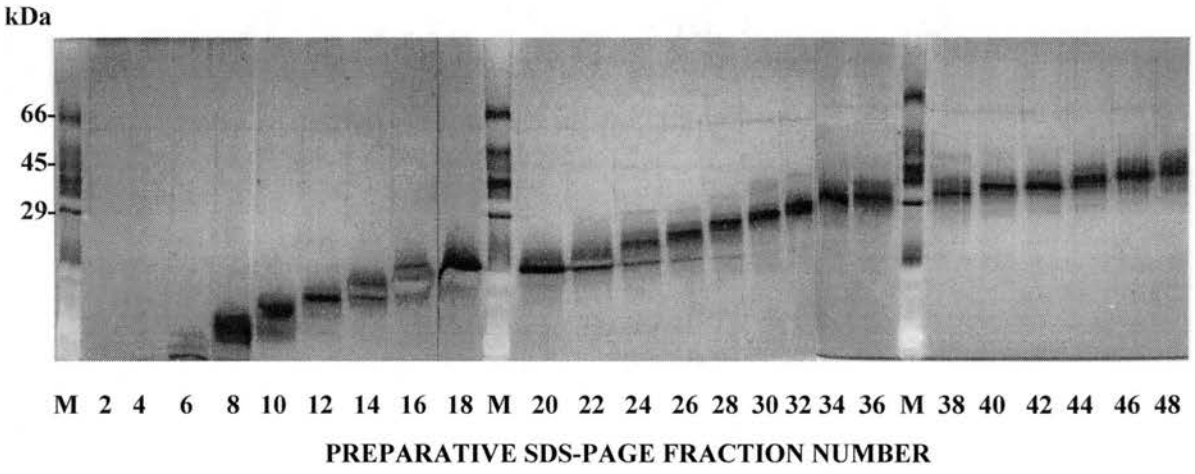
Fractions were electrophoresed in analytical polyacrylamide gel containing 12% acrylamide and stained with silver.
M-Low molecular weight standard

Thus, with a 7.5 % resolving gel, the best preparative fractionation resolution was obtained in the molecular weight range of 45 to 56 kDa.

Fractionation 2: The second fractionation was carried out using a 10 % polyacrylamide resolving gel. Every other fraction, starting with fraction two up to fraction 48 was analyzed for its protein composition using an SDS-PAGE gel containing 12 % acrylamide stained with silver (Figure 2). A very good separation of different molecular weight polypeptides was obtained within the molecular weight range of approximately 10 kDa to 33 kDa. Fractions two and four did not contain any protein bands in it showing that these fractions were collected before the proteins eluted from the resolving gel. Examination of subsequent fractions revealed that gradually higher molecular weight polypeptides eluted as the fractionation progressed. The molecular weight of each fraction was determined to exact value since the purpose was to locate fractions with similar sized proteins so that rational pools could be made.

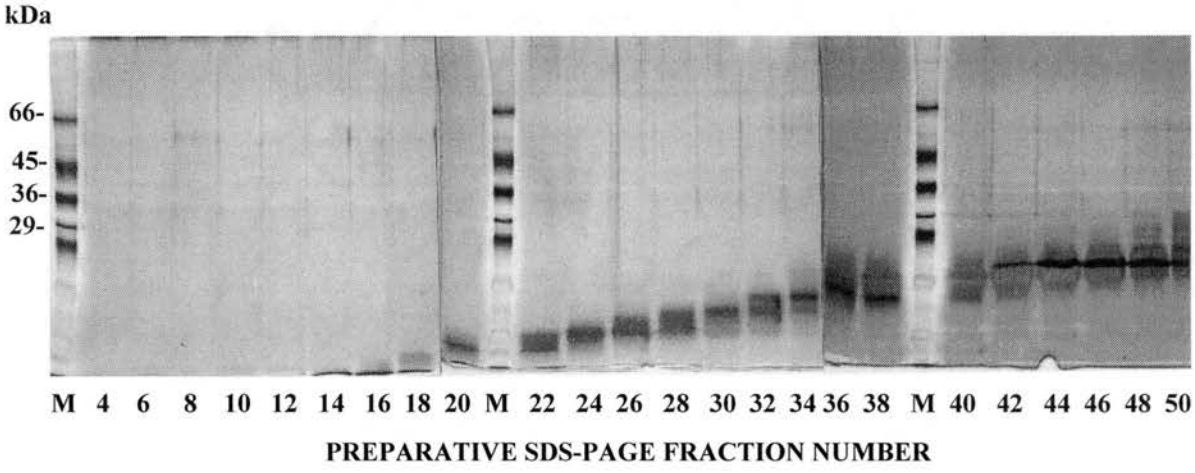
Fractionation 3: The third fractionation was carried out on similar lines as the previous two, using a 12 % polyacrylamide concentration in preparative resolving gel. Figure 3 shows the protein profile of every other fraction, starting with fraction four up to fraction 50 as determined by analytical SDS-PAGE using a 12 % polyacrylamide gel stained with silver. Fractions four, six, eight, 10 and 12 did not show any protein bands meaning that these were collected before any proteins eluted from the resolving gel. Fractions 14, 16, and 18 showed a band at less than 5 kDa. Fractions 20, 22, 24, 26, 28, 30, 32, 34, 36, 38 and 40 showed the appearance of progressively higher molecular weight protein bands in

Figure 2. Preparative scale, continuous-flow, SDS-PAGE fractionation of *Borrelia burgdorferi* (OspA and B deficient strain) whole spirochete extract using 10% acrylamide.



Fractions were electrophoresed in analytical polyacrylamide gel containing 12% acrylamide and stained with silver.
M-Low molecular weight standard

Figure 3. Preparative scale, continuous-flow, SDS-PAGE fractionation of *Borrelia burgdorferi* (OspA and B deficient strain) whole spirochete extract using 12% acrylamide.



Fractions were electrophoresed in analytical polyacrylamide gel containing 12% acrylamide and stained with silver.
M-Low molecular weight standard

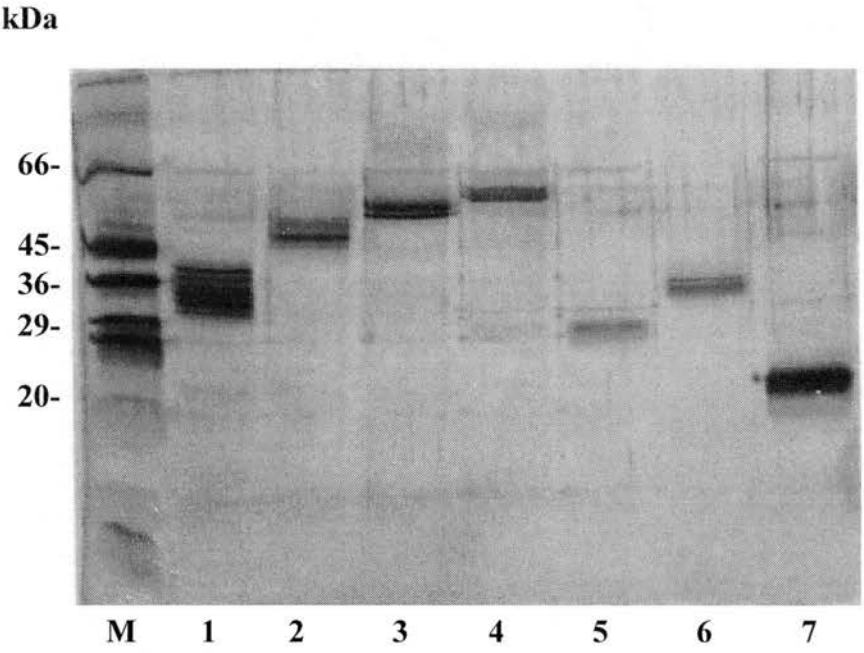
the molecular weight range of approximately 10-20 kDa. However, fractions 42, 44, 46, 48, and 50 contained the same band with a molecular weight of 20 kDa. The best range of resolution with a 12 percent acrylamide concentration in preparative gel was in the range of 10 to 20 kDa.

Pooling of Fractions

After careful analysis of various fractions obtained by the three preparative SDS-PAGE fractionation runs, fractions with similar protein constitution were pooled together to obtain seven different fraction pools. Four separate fraction pools were prepared from the 7.5 % run. Two pools were obtained from the 10 % run. One fraction pool was obtained from the 12 % run. The fractions were pooled with fractions from with in the same run only. After pooling, excess SDS was removed from the pools as described in materials and methods section. Fraction pools were dialyzed against PBS and finally filter sterilized by passing through a 0.22 micron filter. Each fraction pool was analyzed by analytical SDS-PAGE using 12 % polyacrylamide gel stained with silver to determine their protein profile (Figure 4).

Table 1 gives the description of various fraction pools prepared and their protein composition. Fractions five through nine, 14 through 19, 23 through 27, and 30 through 34 obtained from the 7.5 % run were pooled together and were numbered Fraction Pool 1 through 4 (Table 1). Fraction Pool 1 showed six different polypeptide bands ranging in molecular weights from 30.5 to 38.5 kDa (Figure 4). Fraction Pool 2 contained a single major band at 45 kDa (Figure 4). Fraction Pool 3 contained two protein bands at 50 and 51 kDa position (Figure 4). Fraction Pool 4 showed a single major band at the 56 kDa position.

Figure 4. Protein profiles of fraction pools prepared with fractions obtained from three different preparative SDS-PAGE runs on extract from *Borrelia burgdorferi* whole spirochete extract (OspA and B deficient) as determined by analytical SDS-PAGE using 12 % polyacrylamide gel stained with silver.



Lane M is LMW standard (Sigma). Lanes 1 through 7 show constitution of pools 1, 2, 3, 4, 5, 6, and 7 respectively.

Table 1. Description and constitution of fraction pools prepared from different fractions obtained by three different preparative SDS-PAGE runs on WSE.

Fraction Pool No.	Fractions Pooled	Prep run acrylamide %	Mol. Wt. (kDa)
Pool 1	5-9	7.5	30.5-38.5 (6 bands)
Pool 2	14-19	7.5	45
Pool 3	23-27	7.5	50, 51(2 bands)
Pool 4	30-34	7.5	56
Pool 5	32-36	10	27
Pool 6	43-47	10	33
Pool 7	45-49	12	20

Fraction Pools 5 and 6 were obtained by pooling fractions 32 through 36 and 43 through 47, respectively, obtained from the 10 % preparative fractionation run (Table 1). They contained protein bands at 27 and 33 kDa, respectively (Figure 4). Fractions 45 through 49 obtained from the 12 % fractionation were pooled together to give Fraction Pool 7 which contained a single band at 20 kDa position (Table 1, Figure 4). All these fraction pools in addition to WSE (*B. burgdorferi* type strain B31), WSE (*B. burgdorferi* OspA and OspB deficient) and a recombinant OspA (31 kDa) were tested for their effects on proliferative responses of splenocytes obtained from *B. burgdorferi* infected (by needle inoculation and infected tick infestation) and control mice (PBS injected and infested with pathogen-free *Ixodes scapularis* nymphs).

In vitro Splenocyte Proliferation Assay

The splenocytes obtained from mice infected with Lyme borreliosis either by tick transmission or needle inoculation of *B. burgdorferi* were tested for their *in vitro* proliferative responses to different antigens and mitogens as described in the Material and Methods section. The data presented in this section is in terms of stimulation indices (SI's) calculated by dividing mean counts per minute (CPM) obtained from different treatment wells (antigen/mitogen) by the mean CPM obtained from respective control wells i.e. cells incubated in medium alone. An SI of four and above was considered significant proliferation. The actual mean CPM ± S.E obtained are presented in similar tables in Appendix 2.

One week post infection, three day assay: Table 2 shows the SI's obtained in a three day assay performed on splenocytes obtained from mice with tick-transmitted and needle

inoculated infection of Lyme borreliosis, to determine their proliferative responses to different mitogens and antigens. Matched controls infested with pathogen free ticks and injected with PBS were also employed. The actual CPM ± S.E. for this experiment are provided in Table A (Appendix 2).

On incubation with T cell mitogen Con A, an SI of 44.77 was obtained for splenocytes from mice with tick-transmitted Lyme borreliosis which was 17.89 % less proliferative response as compared to the splenocytes from matched control group of mice with pathogen-free tick infestation (SI 54.53). The proliferative response of splenocytes from mice with needle inoculated infection was 52.76 % lower (SI 26.15) than their matched control i.e. splenocytes from mice injected with PBS (SI 55.35), and 41.59 % lower than that of splenocytes from mice with tick transmitted infection.

The proliferative response of splenocytes, from mice with needle inoculated infection, to B-cell mitogen LPS was also suppressed (25.79 %, SI 5.64) as compared to the matched control i.e. PBS injected (SI 7.6). The LPS stimulation indices for splenocytes from mice with tick-transmitted infection and infestation with pathogen free ticks were 8.0 and 7.16, respectively.

The whole spirochete extracts from both B31 strain (WSE B31) and OspA and B negative mutant (WSE AB deficient) caused the splenocytes from all the groups to proliferate significantly. Upon incubation with WSE (B31), SI's of 10.76, 9.62, 12.32, and 12.56 were obtained for splenocytes from mice with tick transmitted infection, needle inoculated infection, pathogen free tick infestation, and PBS injection, respectively. In the same order, SI's obtained upon incubation of splenocytes from respective groups with

Table 2. *In vitro* proliferative responses of splenocytes from BALB/c mice infected with *B. burgdorferi*, either by tick transmission or by needle inoculation, to Concanavalin A (Con A), Lipopolysaccharide (LPS), whole spirochete extract from type strain B31 (WSE B31), whole spirochete extract from OspA and B negative mutant (WSE AB def.), Fraction Pools from preparative SDS-PAGE of WSE (AB def., Pool 1 through 7), and recombinant OspA (Rec. OspA). One week post-infection, three day assay

Treatment	Tick trans. Inf.	Needle inoc. inf.	Uninfected ticks	PBS inject.
Con A	44.77	26.15	54.53	55.35
LPS	8.00	5.64	7.16	7.6
Cells Alone	1.00	1.00	1.00	1.00
WSE (B31)	10.76	9.62	12.32	12.56
WSE (AB def.)	11.17	10.69	13.21	11.04
Pool 1	2.71	1.79	1.44	2.64
Pool 2	0.92	0.90	0.67	1.19
Pool 3	2.79	1.57	1.84	3.02
Pool 4	0.93	0.97	0.72	1.41
Pool 5	1.42	0.81	0.66	1.32
Pool 6	0.85	0.83	0.62	1.19
Pool 7	13.69	4.60	6.68	11.44
Rec. OspA	1.70	2.06	1.32	2.09

5×10^5 splenocytes were incubated with respective antigen/mitogen or without stimulation (cells alone) in triplicate for 54 hrs and then pulsed with one μCi of ^{3}H-thymidine per well. Cells were harvested 18 hrs later and radioactivity incorporation determined by counting in liquid scintillation counter. Data is presented as stimulation indices obtained by dividing the mean CPM obtained from different treatment wells by mean CPM obtained from control wells with cells incubated with medium only.

Splenocytes from mice infested with pathogen free ticks or injected with PBS served as matched controls for tick transmitted and needle inoculated infection, respectively.

WSE (AB deficient) were 11.17, 10.69, 13.21, and 11.04. Slight suppression in responses of groups infected with Lyme borreliosis was observed to both WSE's as compared to their respective matched controls.

Fraction Pools 1 through 6 and recombinant OspA failed to induce significant proliferative response in splenocytes from any group of mice (SI <4). However, Fraction Pool 7 caused significant proliferation of splenocytes from all the groups. An SI of 13.69 was obtained for splenocytes from mice with tick transmitted infection meaning a 51.20 % higher proliferation as compared to the matched control i.e. splenocytes from mice with pathogen free tick infestation (SI 6.68). In contrast, upon incubation with Fraction Pool 7, the proliferative responses of splenocytes from mice with needle inoculated infection were 59.80 % lower (SI 4.60) as compared to their matched control i.e. PBS injected mice (SI 11.44). Overall, with all antigens/mitogens, the proliferative responses of splenocytes from mice with needle inoculated infection were suppressed as compared to other groups.

One week post infection, five day assay: The stimulation indices for all treatments were lower than the respective number in the three day assay (Table 3). The actual mean CPM±S.E obtained are shown in Table B (Appendix 2). The Con A stimulation indices for splenocytes from mice with tick transmitted infection, needle inoculated infection, pathogen free tick infestation, PBS injection were 42.54, 23.15, 45.06, and 47.40, respectively. This shows a respective reduction of 4.98, 11.47, 17.37, and 14.36 % in proliferative responses observed in the three day assay. Significant proliferation was no longer seen in the case of LPS stimulation of splenocytes from mice with needle

Table 3. *In vitro* proliferative responses of splenocytes from BALB/c mice infected with *B. burgdorferi*, either by tick transmission or by needle inoculation, to Concanavalin A (Con A), Lipopolysaccharide (LPS), whole spirochete extract from type strain B31 (WSE B31), whole spirochete extract from OspA and B negative mutant (WSE AB def.), fraction pools from preparative SDS-PAGE of WSE (AB def., Pool 1 through 7), and recombinant OspA (Rec. OspA). One week post-infection, five day assay

Treatment	Tick trans. Inf.	Needle inoc. inf.	Uninfected ticks	PBS inject.
Con A	42.54	23.15	45.06	47.40
LPS	4.69	2.95	2.91	4.07
Cells Alone	1.00	1.00	1.00	1.00
WSE (B31)	2.99	3.95	3.42	2.18
WSE (AB def.)	3.05	4.03	3.24	2.91
Pool 1	1.24	1.16	0.81	1.19
Pool 2	1.21	1.35	0.98	1.27
Pool 3	1.32	1.11	0.92	1.28
Pool 4	0.64	1.36	1.24	1.23
Pool 5	0.67	1.40	1.14	1.27
Pool 6	1.54	1.18	0.92	1.06
Pool 7	0.76	2.04	2.7	2.8
Rec. OspA	1.62	0.91	0.47	0.50

5×10^5 splenocytes were incubated with respective antigen/mitogen or without stimulation (cells alone) in triplicate for 102 hrs and then pulsed with one μCi of ^{3}H-thymidine per well. Cells were harvested 18 hrs later and radioactivity incorporation determined by counting in liquid scintillation counter. Data is presented as stimulation indices obtained by dividing the mean CPM obtained from different treatment wells by mean CPM obtained from control wells with cells with cells incubated with medium only.

Splenocytes from mice infested with pathogen free ticks or injected with PBS served as matched controls for tick transmitted and needle inoculated infection, respectively.

inoculated infection and pathogen free tick infestation. Barely significant proliferation with LPS was seen in splenocytes from mice with tick transmitted infection and those injected with PBS (SI's 4.69 and 4.07, respectively). None of the other SI's obtained were significant except for a 4.03 for splenocytes from mice with needle inoculated infection incubated with WSE (AB def.).

The urinary bladders from all mice were individually cultured in BSK II. After one week of incubation wet smears from these cultures were observed by dark field microscopy. Culture from all the mice that were either infested with infected ticks or given spirochetes IP were found to be positive for infection with *B. burgdorferi*. All the control animals were negative for the presence of spirochetes in their urinary bladder culture.

Two weeks post infection, three day assay: The stimulation indices obtained following a three day *in vitro* proliferation assay on splenocytes obtained from mice two weeks after they were given tick transmitted or needle inoculated infection with Lyme borreliosis are shown in table 4. The actual mean CPM ± S.E. obtained for this experiment are shown in table C (Appendix 2).

A Con A SI of 25.80 was observed for splenocytes from mice with tick transmitted infection, which is, respectively, 63.66, 59.21, and 52.89 % less proliferation as compared to splenocytes from mice with needle inoculated infection (SI 70.99), pathogen free tick infestation (SI 63.25), and PBS injection (SI 54.77).The LPS stimulation index for proliferative response of splenocytes from mice with tick-

Table 4. *In vitro* proliferative responses of splenocytes from BALB/c mice infected with *B. burgdorferi*, either by tick transmission or by needle inoculation, to Concanavalin A (Con A), Lipopolysaccharide (LPS), whole spirochete extract from type strain B31 (WSE B31), whole spirochete extract from OspA and B negative mutant (WSE AB def.), fraction pools from preparative SDS-PAGE of WSE (AB def., Pool 1 through 7), and recombinant OspA (Rec. OspA). Two weeks post-infection, three day assay

Treatment	Tick trans. inf.	Needle inoc. inf.	Uninfected ticks	PBS inject.
Con A	25.80	70.99	63.25	54.77
LPS	4.54	8.09	11.21	9.36
Cells Alone	1.00	1.00	1.00	1.00
WSE (B31)	7.77	12.19	10.40	9.73
WSE (AB def.)	7.55	14.52	12.67	8.69
Pool 1	2.51	3.39	2.87	3.22
Pool 2	0.63	1.15	0.91	1.12
Pool 3	1.97	3.82	3.92	2.55
Pool 4	1.36	1.74	0.91	1.41
Pool 5	1.10	1.12	1.05	1.10
Pool 6	0.67	1.00	0.96	0.86
Pool 7	7.90	13.68	15.08	12.36
Rec. OspA	1.35	2.72	2.19	1.84

5×10^5 splenocytes were incubated with respective antigen/mitogen or without stimulation (cells alone) in triplicate for 54 hrs and then pulsed with one μCi of ^{3}H-thymidine per well. Cells were harvested 18 hrs later and radioactivity incorporation determined by counting in liquid scintillation counter. Data is presented as stimulation indices obtained by dividing the mean CPM obtained from different treatment wells by mean CPM obtained from control wells with cells incubated with medium only.

Splenocytes from mice infested with pathogen free ticks or injected with PBS served as matched controls for tick transmitted and needle inoculated infection, respectively.

transmitted infection was 4.54, which means a respective suppression of 43.88, 59.50, and 51.50 % as compared to splenocytes from mice with needle inoculated infection (SI 8.09), pathogen free tick infestation (SI 11.21) and PBS injection (SI 9.36).

Significant proliferative responses (SI>4) were mounted in response to both whole spirochete extracts i.e. WSE (B31) and WSE (AB def.) by all groups of mice. The response of splenocytes from mice with tick transmitted infection to WSE (B31) and WSE AB def.) was 25.29 and 40.41 % lower (SI 7.77 and 7.55, respectively) as compared to response of splenocytes from matched control that was given a pathogen free tick infestation (SI 10.40 and 12.67, respectively). As in the case of responses to mitogens Con A and LPS, the proliferative responses of splenocytes from mice with needle inoculated infection to WSE (B31) and WSE (AB def.) were higher by 20.18 and 40.15 % (SI 12.19 and 14.52) as compared to responses of the matched control i.e. splenocytes from mice with PBS injection where SI 9.73 and 8.69 were recorded to WSE (B31) and WSE (AB def.), respectively.

The only other antigen which caused the splenocytes to proliferate significantly was the Fraction Pool 7. The respective SI's were 7.90, 13.68, 15.08, and 12.36 for splenocytes from mice with tick transmitted infection, needle inoculated infection, pathogen free tick infestation, and PBS injection. The proliferative responses of splenocytes from mice with tick transmitted infection to Fraction Pool 7 were respectively 42.25 and 47.61 % less as compared to splenocyte from mice with needle inoculated infection and those with pathogen free tick infestation.

Two weeks post infection, five day assay: The results (as SI's) of the five day splenocyte proliferation assay conducted at two weeks post infection are shown in table 5.

Table 5. *In vitro* proliferative responses of splenocytes from BALB/c mice infected with *B. burgdorferi*, either by tick transmission or by needle inoculation, to Concanavalin A (Con A), Lipopolysaccharide (LPS), whole spirochete extract from type strain B31 (WSE B31), whole spirochete extract from OspA and B negative mutant (WSE AB def.), fraction pools from preparative SDS-PAGE of WSE (AB def., Pool 1 through 7), and recombinant OspA (Rec. OspA). Two weeks post-infection, five day assay

Treatment	Tick trans. inf.	Needle inoc. inf.	Uninfected ticks	PBS inject.
Con A	22.99	36.24	47.24	71.35
LPS	3.10	3.57	6.65	8.95
Cells Alone	1.00	1.00	1.00	1.00
WSE (B31)	3.17	3.75	3.07	3.00
WSE (AB def.)	3.45	4.88	3.21	3.38
Pool 1	2.11	3.28	2.08	2.44
Pool 2	1.58	0.76	0.80	0.50
Pool 3	1.61	3.53	2.16	2.42
Pool 4	3.48	0.96	0.65	0.64
Pool 5	1.49	0.94	0.71	0.90
Pool 6	0.68	0.63	0.74	0.49
Pool 7	3.92	5.78	5.31	9.23
Rec. OspA	0.55	1.31	0.48	0.55

5×10^5 splenocytes were incubated with respective antigen/mitogen or without stimulation (cells alone) in triplicate for 102 hrs and then pulsed with one μCi of ^{3}H-thymidine per well. Cells were harvested 18 hrs later and radioactivity incorporation determined by counting in liquid scintillation counter. Data is presented as stimulation indices obtained by dividing the mean CPM obtained from different treatment wells by mean CPM obtained from control wells with cells incubated with medium only.

Splenocytes from mice infested with pathogen free ticks or injected with PBS served as matched controls for tick transmitted and needle inoculated infection, respectively.

The actual mean CPM ± S.E. obtained for this experiment are given in table D (Appendix 2). Significant proliferative responses to Con A were seen in all groups where SI's of 22.99, 36.24, 47.24, and 71.35 were obtained for splenocytes from mice with tick transmitted infection, needle inoculated infection, pathogen free tick infestation, and PBS injection, respectively. However, splenocytes from mice with tick transmitted infection and needle inoculated infection no longer showed significant proliferation in response to LPS (SI's<4). The matched controls i.e. splenocytes from mice with pathogen free tick infestation and PBS injection showed significant proliferation in response to LPS (SI's 6.65 and 8.95) but lower as compared to three day assay (respective SI's 11.21 and 9.36).

A barely significant (SI 4.88), proliferative response of splenocytes from mice with needle inoculated infection was observed in response to WSE (AB def.). The splenocytes from other groups did not respond significantly to stimulation with either of the WSE's. The only other significant proliferative responses observed were upon stimulation with Fraction Pool 7 in splenocytes from mice with needle inoculated infection, pathogen free tick infestation and PBS injection (SI's 5.78, 5.31, and 9.23, respectively). These responses were 57.75, 64.79, and 25.32 % lower than the respective responses in the three day assay.

Once again, urinary bladder cultures from mice given infection by either route were positive for the presence of spirochetes after one week of incubation in BSK II. All the control mice were negative for the infection.

Four weeks post infection, three day and five day assay: The results of both three day and five day *in vitro* splenocyte proliferation assay at four weeks post infection are shown

in Table 6 as stimulation indices. The actual mean CPM ± S.E. obtained are shown in table E (Appendix 2). As explained in material and method (experimental design) section, the matched controls i.e. groups of mice with pathogen free tick infestation and PBS injection were not employed at this interval.

In the three day assay, the proliferative response of splenocytes from mice with tick transmitted infection to T cell mitogen Con A (SI 31.08) was 44.22 % less than that of splenocytes from mice with needle inoculated infection (SI 55.72). A similar observation was made for the response of both groups in the five day assay where the Con A SI's were 32.54 and 60.04, respectively for splenocytes from mice with tick transmitted infection and needle inoculated infection. However, the proliferative response of splenocytes from mice with tick transmitted infection group and needle inoculated infection group to B cell mitogen LPS was almost same (SI 5.70 and 5.61, respectively) in the three day assay. However, in the five day assay, the proliferative response (to LPS) of splenocytes from mice with tick transmitted infection was no longer significant (SI 2.86) and those of needle inoculated infection were only slightly significant (SI 4.08).

In the three day assay, the splenocytes from mice with tick transmitted infection upon stimulation with WSE (B31) and WSE (AB def.) showed SI's of 6.79 and 5.99, respectively. These responses were 39.70 and 43.11 % lower than those for splenocytes from mice with needle inoculated infection which showed SI's of 11.26 and 10.53 upon stimulation with WSE (B31) and WSE (AB def.). However, in the five day assay, proliferative responses of splenocytes from mice with tick transmitted infection to either of the WSE's were no longer significant. In contrast, proliferative response of splenocytes

Table 6. *In vitro* proliferative responses of splenocytes from BALB/c mice infected with *B. burgdorferi*, either by tick transmission or by needle inoculation, to Concanavalin A (Con A), Lipopolysaccharide (LPS), whole spirochete extract from type strain B31 (WSE B31), whole spirochete extract from OspA and B negative mutant (WSE AB def.), fraction pools from preparative SDS-PAGE of WSE (AB def., Pool 1 through 7), and recombinant OspA (Rec. OspA). Four weeks post-infection, three and five day assay

	Three day assay		Five day assay	
Treatment	**Tick trans. inf.**	**Needle inoc. inf.**	**Tick trans. inf.**	**Needle inoc. inf.**
Con A	31.08	55.72	32.54	60.04
LPS	5.70	5.61	2.86	4.08
Cells Alone	1.00	1.00	1.00	1.00
WSE (B31)	6.79	11.26	2.01	4.09
WSE (AB def.)	5.99	10.53	2.06	5.11
Pool 1	2.48	4.08	2.08	2.67
Pool 2	1.22	1.77	1.30	1.87
Pool 3	3.02	4.35	1.47	3.08
Pool 4	1.51	2.42	1.44	3.41
Pool 5	1.28	1.50	1.13	1.29
Pool 6	1.18	2.01	1.62	1.82
Pool 7	11.28	19.54	7.67	10.57
Rec. OspA	3.28	2.57	1.28	1.08

5×10^5 splenocytes were incubated with respective antigen/mitogen or without stimulation (cells alone) in triplicate for 54 and 102 hrs (for three day and five day assay, respectively) and then pulsed with one μCi of ^{3}H-thymidine per well. Cells were harvested 18 hrs later and radioactivity incorporation determined by counting in liquid scintillation counter. Data is presented as stimulation indices obtained by dividing the mean CPM obtained from different treatment wells by mean CPM obtained from control wells where cells were incubated with medium only.
Splenocytes from mice infested with clean ticks or injected with PBS which served as matched controls for tick transmitted and needle inoculated infection, respectively, were not done.

from needle inoculated infection to WSE (B31) and WSE (AB def.) were significant (SI's 4.09 and 5.11, respectively).

In the three day assay, a significant proliferation was seen in response to Fraction Pools 1 and 3 (SI's 4.08 and 4.35, respectively) in the case of splenocytes from mice with needle inoculated infection. A similar response was not seen in the case of splenocytes from mice with tick transmitted infection. This response disappeared even for splenocytes from needle inoculated infection in the five day assay.

In the three day assay, splenocytes from mice with tick transmitted infection proliferated 42.27 % less as compared to the splenocytes from mice with needle inoculated infection upon incubation with Fraction Pool seven (respective SI's 11.28 and 19.54). Surprisingly, the proliferation levels in response to Fraction Pool 7 for splenocytes from both groups of mice were much higher even as compared to stimulation with either of WSE's. In the five day assay, the proliferative response of splenocytes to Fraction Pool 7 was still significant with SI's 7.67 and 10.57, for tick transmitted infection and needle inoculated infection groups, respectively, although lower than in the three day assay. All the mice were confirmed to be positive for *B. burgdorferi* infection by urinary bladder culture.

Eight weeks post infection, three day assay: The results of this experiment in terms of stimulation indices are shown in table 7. The actual mean CPM ± S.E. obtained are presented in table F (Appendix 2). As explained in the material and method (experimental design) section, Fraction Pools 1 through 6 and recombinant OspA were not tested at this

Table 7. *In vitro* proliferative responses of splenocytes from BALB/c mice infected with *B. burgdorferi*, either by tick transmission or by needle inoculation, to Concanavalin A (Con A), Lipopolysaccharide (LPS), whole spirochete extract from type strain B31 (WSE B31), whole spirochete extract from OspA and B negative mutant (WSE AB def.), and Fraction Pool 7 obtained by preparative SDS-PAGE of WSE (AB def.). Eight weeks post-infection, three day assay

Treatment	Tick trans. inf.	Needle inoc. inf.	Uninfected ticks	PBS inject.
Con A	84.86	141.35	87.34	93.72
LPS	31.43	75.04	36.73	45.74
Cells Alone	1.00	1.00	1.00	1.00
WSE (B31)	25.09	66.20	29.10	31.54
WSE (AB def.)	15.30	30.43	19.33	15.49
Pool 7	24.56	45.04	20.93	24.76

5×10^5 splenocytes were incubated with respective antigen/mitogen or without stimulation (cells alone) in triplicate for 54 hrs and then pulsed with one μCi of ^{3}H-thymidine per well. Cells were harvested 18 hrs later and radioactivity incorporation determined by counting in liquid scintillation counter. Data is presented as stimulation indices obtained by dividing the mean CPM obtained from different treatment wells by mean CPM obtained from control wells with cells incubated with medium alone.

Splenocytes from mice infested with pathogen free ticks or injected with PBS served as matched controls for tick transmitted and needle inoculated infection, respectively. Fraction Pools one through six and recombinant OspA were not tested.

interval. Also, only two mice instead of four were used in each control group i.e. pathogen free tick infested and PBS injected.

The level of proliferation of splenocytes from mice with tick transmitted infection in response to Con A and LPS (SI's 84.86 and 31.43, respectively) was almost same as for splenocytes from mice with pathogen free tick infestation (SI's 87.34 and 36.73, respectively for Con A and LPS). However, splenocytes from mice with needle inoculated infection proliferated to a much higher degree (SI 141.35 for Con A and 75.04 for LPS) as compared to the matched control of splenocytes from mice with PBS injection which showed SI 93.72 with Con A (33.70 % less) and SI 45.74 with LPS (39.04 % less). Also, response of splenocytes from mice with needle inoculated infection was 39.96 % and 58.11 % higher as compared to splenocytes from mice with tick transmitted infection upon incubation with Con A and LPS, respectively.

The SI's obtained upon incubation with WSE (B31) were 25.09, 66.20, 29.10, and 31.54, respectively for splenocytes from mice with tick transmitted infection, needle inoculated infection, pathogen free tick infestation, and PBS injection. With WSE (AB def.) the respective SI's were 15.30, 30.43, 19.33, and 15.49. The response of splenocytes from tick transmitted infection to WSE (B31) and WSE (AB def.) was almost same or slightly lower than that of matched control of splenocytes from mice with pathogen free tick infestation. However, the splenocytes from mice with needle inoculated infection responded 52.36 and 49.10 % higher than the splenocytes from the matched control i.e. mice with PBS injection in response to WSE (B31) and WSE (AB def.), respectively. Similarly, the response of splenocytes from mice with needle inoculated infection was

62.09 and 49.72 % higher than that of splenocytes from mice with tick transmitted infection, to WSE (B31) and WSE (AB def.), respectively.

Incubation with Fraction Pool 7 resulted in significant proliferation of splenocytes from all groups of mice. The SI's obtained were 24.56 for mice with tick transmitted infection, 45.04 for mice with needle inoculated infection, 20.93 for mice with pathogen free tick infestation, and 24.76 for mice with PBS injection. The response of splenocytes from mice with needle inoculated infection was 45.03 % higher than the matched control of splenocytes from mice with PBS injection and 45.47 % higher than splenocytes from mice with tick transmitted infection. The response of splenocytes from mice with tick transmitted infection was slightly higher (14.78 %) as compared to the matched control of splenocytes from mice with pathogen free tick infestation.

Eight weeks post infection, five day assay: The results of this experiment in terms of stimulation indices are shown in table 8. The actual mean CPM ± S.E. obtained are presented in table G (Appendix 2). As explained in the Material and Method (experimental design) section, Fraction Pools 1 through 6 and recombinant OspA were not tested at this interval.

The SI's upon incubation of splenocytes from mice with tick transmitted infection, needle inoculated infection, pathogen free tick infestation, and PBS injection were 46.69, 50.45, 55.02, and 54.51, respectively. The respective SI's for LPS were 4.24, 9.81, 6.03 and 10.00. The proliferation level of splenocytes from mice with tick transmitted infection and PBS injection were no longer significant in response to incubation with WSE (B31). The SI's for splenocytes from mice with needle inoculated

Table 8. *In vitro* proliferative responses of splenocytes from BALB/c mice infected with *B. burgdorferi*, either by tick transmission or by needle inoculation, to Concanavalin A (Con A), Lipopolysaccharide (LPS), whole spirochete extract from type strain B31 (WSE B31), whole spirochete extract from OspA and B negative mutant (WSE AB def.), and Fraction Pool 7 obtained by preparative SDS-PAGE fractionation of WSE (AB def.). Eight weeks post-infection, five day assay

Treatment	Tick trans. inf.	Needle inoc. inf.	Uninfected ticks	PBS inject.
Con A	46.69	50.45	55.02	54.51
LPS	4.24	9.81	6.03	10.00
Cells Alone	1.00	1.00	1.00	1.00
WSE (B31)	2.90	13.80	4.32	3.19
WSE (AB def.)	1.96	4.89	2.56	1.70
Pool 7	2.79	5.31	2.77	1.86

5×10^5 splenocytes were incubated with respective antigen/mitogen or without stimulation (cells alone) in triplicate for 102 hrs and then pulsed with one μCi of ^{3}H-thymidine per well. Cells were harvested 18 hrs later and radioactivity incorporation determined by counting in liquid scintillation counter. Data is presented as stimulation indices obtained by dividing the mean CPM obtained from different treatment wells by mean CPM obtained from control wells with cells incubated with medium only.

Splenocytes from mice infested with pathogen free ticks or injected with PBS served as matched controls for tick transmitted and needle inoculated infection, respectively. Fraction pools one through six and recombinant OspA were not tested.

infection and pathogen free tick infestation were respectively 13.8 and 4.32 in response to WSE (B31).

The splenocytes from mice with needle inoculated infection showed significant proliferation in response to incubation with WSE (AB def.) and Fraction Pool 7. The respective SI's were 4.89 and 5.31 respectively. The splenocytes from the other three groups did not show significant proliferation in response to either WSE (AB def.) or Fraction Pool 7.

All the mice infected by either needle inoculation or tick transmission were positive for infection as determined by the urinary bladder culture. All the control mice were negative for infection.

12 weeks post infection, three day assay: The results of this experiment in terms of SI's are shown in table 9. The actual mean CPM ± S.E. obtained are presented in table H (Appendix 2). The proliferative response of splenocytes from mice with tick transmitted infection showed an SI of 44.47 as compared to an SI 97.61 (54.44 % higher) obtained with matched control of splenocytes from mice with pathogen free tick infestation. The splenocytes from mice with needle inoculated infection proliferated almost to the same level as matched control of splenocytes from mice with PBS injection. The respective SI's were 61.21 and 66.72. The proliferation of splenocytes from all the groups was almost to the same level in response to incubation with LPS where SI's 4.98, 6.04, 6.42, and 5.76, respectively were obtained for splenocytes from mice with tick transmitted infection, needle inoculated infection, pathogen free tick infestation, and PBS injection.

Table 9. *In vitro* proliferative responses of splenocytes from BALB/c mice infected with *B. burgdorferi*, either by tick transmission or by needle inoculation, to Concanavalin A (Con A), Lipopolysaccharide (LPS), whole spirochete extract from type strain B31 (WSE B31), whole spirochete extract from OspA and B negative mutant (WSE AB def.), fraction pools from preparative SDS-PAGE of WSE (AB def., Pool 1 through 7), and recombinant OspA (Rec. OspA). 12 weeks post-infection, three day assay

Treatment	Tick trans. inf.	Needle inoc. inf.	Uninfected ticks	PBS inject.
Con A	44.47	61.21	97.61	66.72
LPS	4.98	6.04	6.42	5.76
Cells Alone	1.00	1.00	1.00	1.00
WSE (B31)	10.61	16.25	13.99	16.09
WSE (AB def.)	7.32	14.05	6.35	8.76
Pool 1	1.03	0.97	1.24	1.39
Pool 2	0.90	1.07	0.98	1.27
Pool 3	1.12	1.55	1.39	1.75
Pool 4	0.64	0.95	0.85	1.00
Pool 5	0.82	0.93	1.07	1.31
Pool 6	0.61	1.02	0.85	6.32
Pool 7	3.39	6.52	6.59	1.21
Rec. OspA	2.59	4.77	4.19	4.68

5×10^5 splenocytes were incubated with respective antigen/mitogen or without stimulation (cells alone) in triplicate for 54 hrs and then pulsed with one μCi of ^{3}H-thymidine per well. Cells were harvested 18 hrs later and radioactivity incorporation determined by counting in liquid scintillation counter. Data is presented as stimulation indices obtained by dividing the mean CPM obtained from different treatment wells by mean CPM obtained from control wells with cells incubated in medium only.

Splenocytes from mice infested with pathogen free ticks or injected with PBS served as matched controls for tick transmitted and needle inoculated infection, respectively.

In the case of stimulation with WSE (B31), the proliferation response of splenocytes from mice with tick transmitted infection (SI 10.61) was slightly lower (24.16 %) than the matched control of splenocytes from mice with pathogen free tick infestation (SI 13.99). On the other hand the splenocytes from mice with needle inoculated infection proliferated to the same level as their matched controls i.e. splenocytes from mice with PBS injection and the SI's obtained were 16.25 and 16.09, respectively. Stimulation indices of 7.32 and 6.35 were obtained for splenocytes from mice with tick-transmitted infection and pathogen free tick infestation, respectively upon stimulation with WSE (AB def.) The proliferative response of splenocytes from mice with needle inoculated infection (SI 14.05) was 37.65 % higher than that of the matched control i.e. splenocytes from mice with PBS injection (SI 8.76) to WSE (AB def.).The response of splenocytes from mice with needle inoculated infection was 37.65 % higher as compared to splenocytes from mice with tick transmitted infection upon stimulation with WSE (AB def.).

Fraction Pools one through six did not cause splenocytes from any group to proliferate significantly. The splenocytes from mice with needle inoculated infection and those infested with pathogen free ticks showed SI's 6.52 and 6.59 when incubated with Fraction Pool 7. Surprisingly, splenocytes from either mice with tick transmitted infection or those with PBS injection did not proliferate significantly when stimulated with Fraction Pool 7.

The splenocytes from mice with needle inoculated infection, pathogen free tick infestation, and PBS injection showed SI's 4.77, 4.19, and 4.68 upon incubation with

recombinant OspA. The splenocytes from mice with tick transmitted infection did not proliferate significantly in response to recombinant OspA.

Twelve weeks post infection, five day assay: The SI's deduced for this experiment are presented in table 10. The actual mean CPM ± S.E. obtained are presented in table I (Appendix 2). The proliferative responses to Con A by splenocytes from mice with pathogen free tick infestation (SI 85.81) and those from mice with PBS injection (SI 107.61) were respectively, 22.34 and 27.73 % higher than their matched experimental groups i.e. tick transmitted infection (SI 66.64) and needle inoculated infection (SI 77.77). Stimulation indices obtained following incubation with LPS gave SI's 7.66, 8.35, 9.02, and 6.34 for splenocytes from mice with tick transmitted infection, needle inoculated infection, pathogen free tick infestation, and PBS injection.

The proliferative response upon incubation with WSE (B31) for splenocytes from mice with tick transmitted infection (SI 7.77) was 49.90 % less as compared to the matched control of splenocytes from mice with pathogen free tick infestation (SI 15.51). However, splenocytes from mice with needle inoculated infection responded at slightly higher level than their matched control group of splenocytes from mice with PBS injection (SI's 11.77 and 8.22, respectively). The similar trend between respective groups was maintained for stimulation with WSE (AB def.) where SI's 6.07, 9.01, 10.95, and 8.65 were determined for splenocytes from mice with tick transmitted infection, needle inoculated infection, pathogen free tick infestation, and PBS injection, respectively.

Incubation with Fraction Pools one through six did not cause a significant level of proliferation (all SI's<4) in splenocytes from any group of mice. Fraction Pool 7 caused

Table 10. *In vitro* proliferative responses of splenocytes from BALB/c mice infected with *B. burgdorferi*, either by tick transmission or by needle inoculation, to Concanavalin A (Con A), Lipopolysaccharide (LPS), whole spirochete extract from type strain B31 (WSE B31), whole spirochete extract from OspA and B negative mutant (WSE AB def.), fraction pools from preparative SDS-PAGE of WSE (AB def., Pool 1 through 7), and recombinant OspA (Rec. OspA). 12 weeks post-infection, five day assay

Treatment	Tick trans. inf.	Needle inoc. inf.	Uninfected ticks	PBS inject.
Con A	66.64	77.77	85.81	107.61
LPS	7.66	8.35	9.02	6.34
Cells Alone	1.00	1.00	1.00	1.00
WSE (B31)	7.77	11.77	15.51	8.22
WSE (AB def.)	6.07	9.01	10.95	8.65
Pool 1	0.65	0.80	1.40	0.94
Pool 2	0.59	1.01	0.79	1.09
Pool 3	0.76	1.17	1.71	0.99
Pool 4	0.78	0.85	1.04	0.79
Pool 5	0.63	0.65	0.77	0.63
Pool 6	0.73	0.65	0.82	1.33
Pool 7	3.09	5.13	3.59	4.46
Rec. OspA	1.82	2.84	4.88	3.33

5×10^5 splenocytes were incubated with respective antigen/mitogen or without stimulation (cells alone) in triplicate for 102 hrs and then pulsed with one μCi of ^{3}H-thymidine per well. Cells were harvested 18 hrs later and radioactivity incorporation determined by counting in liquid scintillation counter. Data is presented as stimulation indices obtained by dividing the mean CPM obtained from different treatment wells by mean CPM obtained from control wells with cells incubated in medium only.

Splenocytes from mice infested with pathogen free ticks or injected with PBS served as matched controls for tick transmitted and needle inoculated infection, respectively.

low level proliferation in splenocytes from the group of mice with needle inoculated infection (SI 5.13), and its matched control of splenocytes from mice with PBS injection. The splenocytes from mice with tick transmitted infection and those with pathogen free tick infestation did not proliferate significantly upon incubation with Fraction Pool 7.

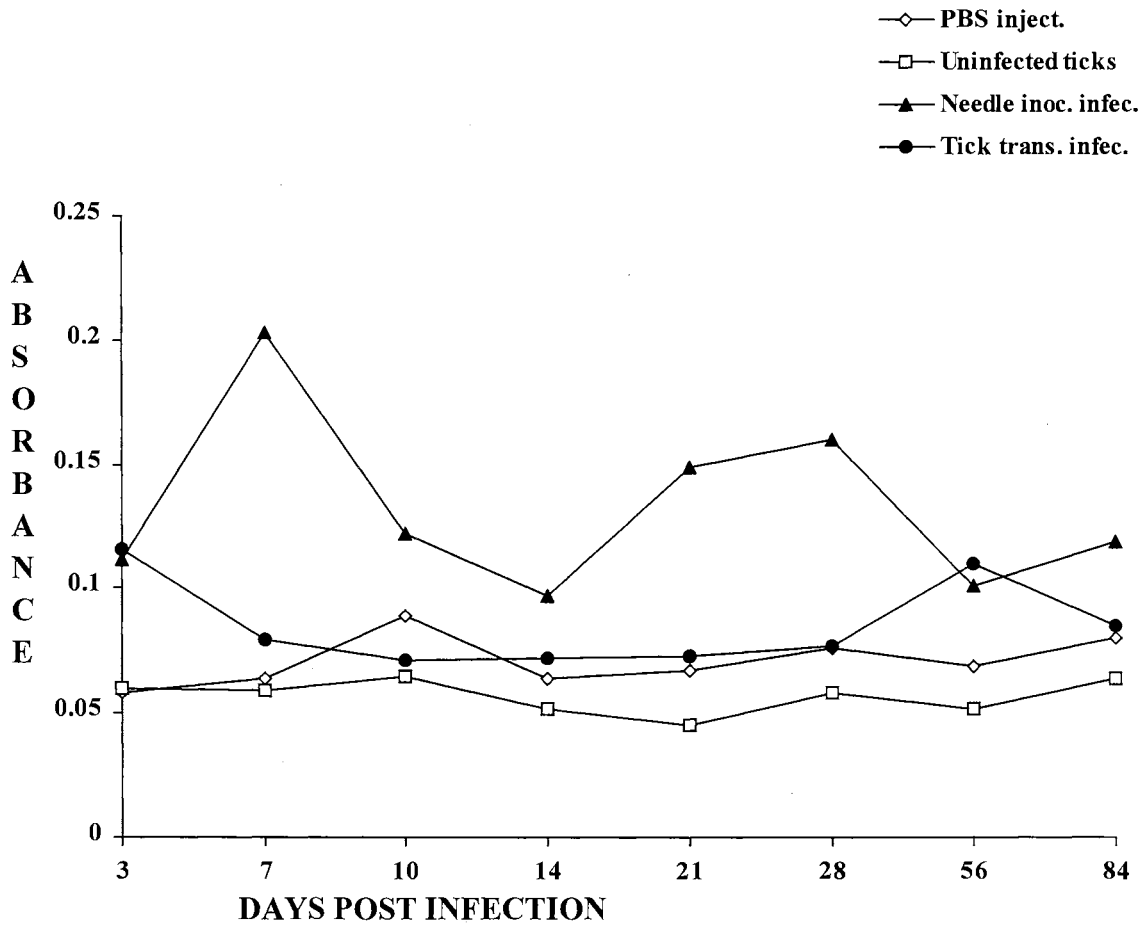
Recombinant OspA caused barely significant proliferation in splenocytes from mice with pathogen free tick infestation only (SI 4.88). All the mice that were given *B. burgdorferi* infection either by tick transmission or by needle inoculation of culture grown spirochetes were confirmed to be positive for infection by urinary bladder culture. The control mice were negative for infection with Lyme borreliosis.

Antibody Response

The serum samples collected at different intervals from groups of mice infected with *B. burgdorferi* (tick transmission and needle inoculation) were examined for the presence of anti-spirochete antibodies. A sensitive ELISA protocol was used to monitor both IgM and IgG isotypes. Sera collected at similar intervals from control mice i.e. pathogen free tick infested and PBS injected served as controls. The sera were available for day three, seven, ten, 14, 21, 28, 56, and 84 post-infection.

The mean absorbance values obtained for the presence of *B. burgdorferi* specific IgM antibodies in the sera collected at different intervals are plotted in Figure 5. The data presented is at a dilution of 1:5120 for all sera. The mean absorbance value obtained for direct binding of second antibody i.e. anti-mouse IgM-HRPO conjugate (at a dilution of 1:5000) to *B. burgdorferi* antigen was 0.036. In the case of needle inoculated infection anti-spirochete IgM antibody was detectable as early as third day post infection (mean absorbance value 0.112) and peaked at day seven (mean absorbance value 0.203).

Figure 5. *Borrelia burgdorferi* specific IgM antibody response in BALB/c mice infected by tick transmission or needle inoculation of the spirochete as determined by enzyme linked immunosorbant assay.

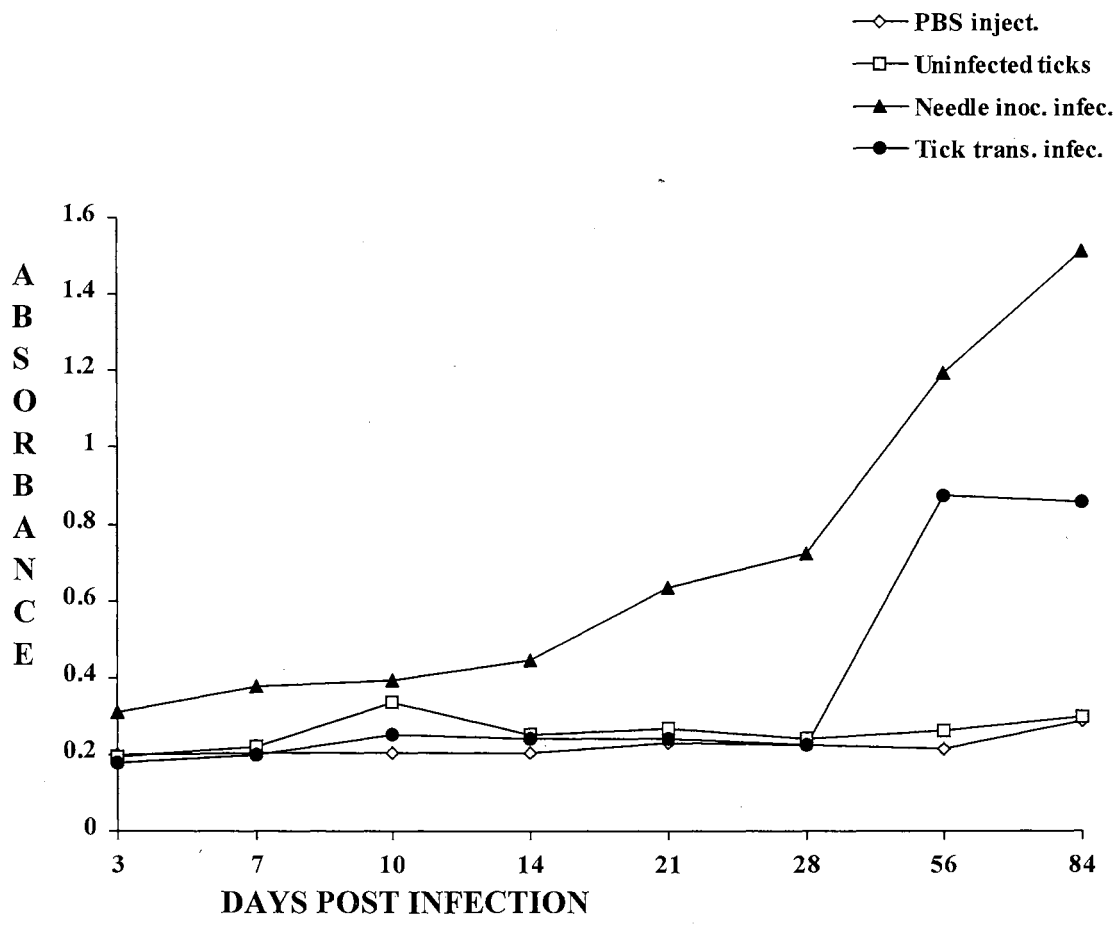


Thereafter, a drop was observed at day 10 and 14 (mean absorbance values 0.122 and 0.097, respectively). On day 21, the anti-spirochete IgM antibody showed a rise (mean absorbance value 0.149) and peaked at 28 days post-infection (mean absorbance value 0.160). On day 56 and 84 post-infection, the mean absorbance values obtained were 0.101 and 0.119, respectively. For the matched control, i.e. sera from mice that received a PBS injection, the mean absorbance values were 0.058, 0.064, 0.089, 0.064, 0.067, 0.076, 0.069, and 0.080 at day three, seven, 10, 14, 21, 28, 56, and 84, respectively.

In the sera collected from mice with tick-transmitted infection, some anti-spirochete IgM antibody was detectable at day three post infection (mean absorbance value 0.116). The mean absorbance value for the serum collected from mice given an infestation with pathogen free ticks (matched control) was 0.058. A very low level of anti-spirochete IgM antibody was detected in sera collected from mice with tick transmitted infection at subsequent intervals as compared to the sera from mice with needle inoculated infection. The mean absorbance values for sera collected from mice with tick transmitted infection at days seven, 10, 14, 21, 28, 56, and 84 were 0.079, 0.071, 0.072, 0.073, 0.077, 0.110, and 0.085. The mean absorbance values at the same intervals for sera from mice with pathogen free tick infestation were 0.059, 0.065, 0.052, 0.045, 0.058, 0.052, and 0.064.

The mean absorbance values obtained for detection of anti-spirochete IgG antibody in sera collected from different groups of mice are plotted in Figure 6. The data used is at 1:20 dilution for all sera. The mean absorbance value for direct binding of

Figure 6. *Borrelia burgdorferi* specific IgG antibody response in BALB/c mice infected by tick transmission or needle inoculation of spirochetes as determined by enzyme linked immunosorbant assay.



second antibody i.e. anti-mouse IgG-HRPO conjugate (1:5000) with *B. burgdorferi* antigen was 0.058.

Anti-spirochete IgG antibody was also detectable as early as three days post infection in the case of sera collected from mice with needle inoculated infection. The mean absorbance value for serum from mice inoculated with needle inoculated infection at three days post infection was 0.309 as compared to serum from mice given PBS injection where the mean absorbance value was 0.200. The sera collected from mice with needle inoculated infection at subsequent intervals thereafter showed a gradual increase in the amount of anti-spirochete IgG antibody. The mean absorbance values were 0.378, 0.392, 0.445, 0.633, 0.723, 1.198, and 1.515 at seven, 10, 14, 21, 28, 56, and 84 days post-infection, respectively. The matched control of sera collected from mice with PBS injection (matched control) showed mean absorbance values of 0.205, 0.202, 0.206, 0.231, 0.224, 0.214, and 0.287 respectively for seven, 10, 14, 21, 28, 56, and 84 days post-infection, respectively.

In contrast to the sera from mice with needle inoculated infection, the sera from mice with tick transmitted infection showed much less titer of anti-spirochete IgG antibody. In fact, no significant anti-spirochete IgG was detectable till day 56 post-infection. The mean absorbance values for sera from mice with tick transmitted infection at day three, seven, 10, 14, 21, and 28 post-infection were 0.180, 0.200, 0.252, 0.243, 0.240, and 0.228 respectively as compared to the matched control of sera from mice with pathogen free tick infestation where mean absorbance values were 0.196, 0.222, 0.337, 0.253, 0.267, and 0.242, respectively at the same time intervals. However, at day 56 and 84, a respective mean absorbance value of 0.876 and 0.862 was recorded, showing the

appearance of anti-spirochete IgG. The mean absorbance values recorded for matched control sera at day 56 and 84 were 0.263 and 0.298 respectively.

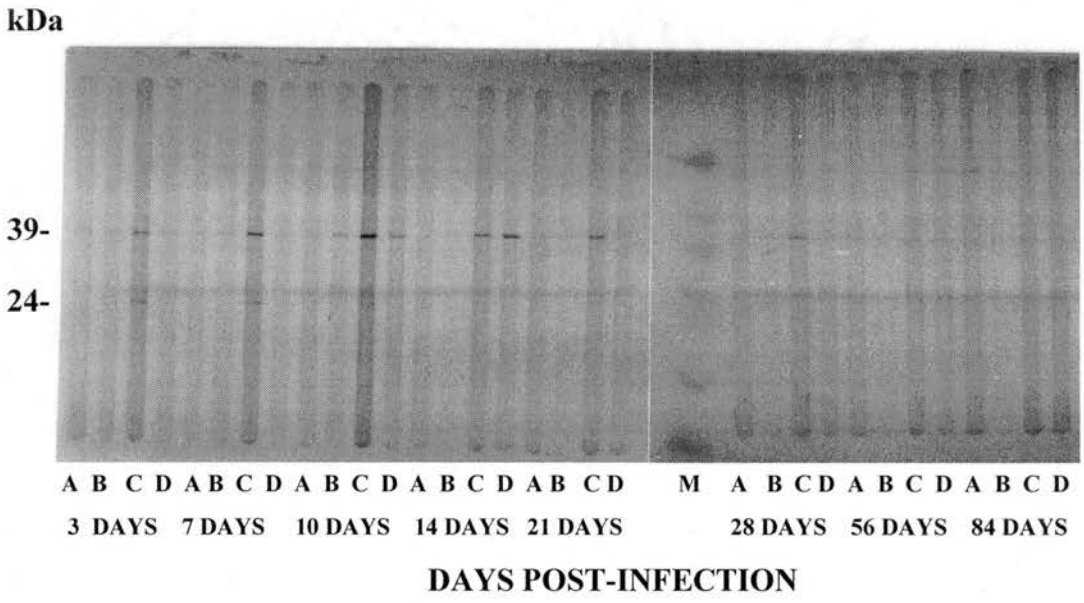
Immunoblot Analysis

All the sera studied by ELISA were also available for Western immunoblot analysis. Once again both IgM and IgG isotype anti-spirochete antibodies were monitored. Anti-spirochete IgM reactive with 39 and 24 kDa spirochetal proteins was detectable as early as three days after infecting by needle inoculation (Figure 7). This reactivity remained detectable at seven, ten, 14 and 21 days post infection. At 28 days post infection reactivity to the 24 kDa protein was lost, however, anti-39 kDa activity was still present (Figure 8). After 28 days post infection no anti-spirochetal IgM antibody was detected in the sera from mice infected by needle inoculation.

At ten days post infection, an IgM reactivity to a 39 kDa protein was evident in serum collected from mice infected by tick transmitted infection and remained detectable on days 14 and 21(Figure 7). Thereafter no anti-spirochetal IgM reactivity was present in these serum samples (Figure 7). None of the control sera showed any reactivity against borrelial proteins.

An IgG reactivity at ten days post infection was detectable against 39 kDa and 31 kDa protein in the sera collected from mice needle inoculated with spirochete (Figure 8) and lasted through the duration of experiment. In contrast, no IgG reactivity against the 31 kDa protein was detectable in the sera collected from mice infected by tick-transmission of the spirochete at any time during the experiment. The only reactivity detected was against the 39 kDa protein that appeared at 28 days post infection and remained detectable through 84 days post infection.

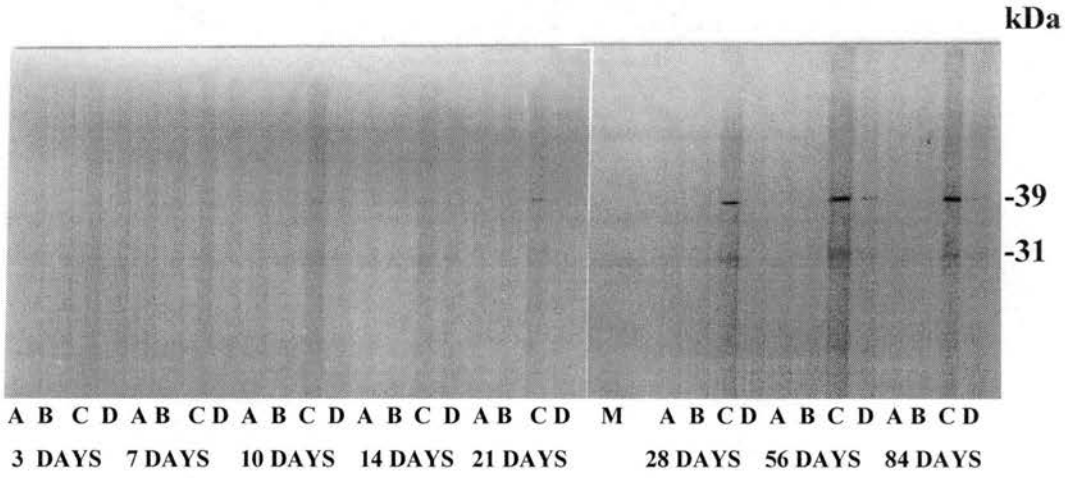
Figure 7. Immunoblot analysis of sera collected at different intervals from infected and control mice for anti-*Borrelia burgdorferi* IgM antibodies



A: PBS injected **B: Pathogen free ticks** **C: Needle inoculated infection**

D: Tick-transmitted infection **M: Molecular weight marker**

Figure 8. Western blot analysis of sera collected at different intervals from infected and control mice for anti-*Borrelia burgdorferi* IgG antibodies



A: PBS injected B: Pathogen free ticks C: Needle inoculated infection

D: Tick-transmitted infection M: Molecular weight marker

In vitro Spirochete Neutralization Assay

Various sera collected at different intervals i.e. three, seven, ten, 14, 21, 28, 56 and 84 days post infection, from mice infected by needle inoculation or tick transmission of *B. burgdorferi* tested for their ability to kill the spirochetes *in vitro*. The sera collected from clean tick infested and PBS injected mice at three, 28, and 84 day interval served as negative controls.

Spirochete neutralization activity appeared as early as three days post infection in serum collected from mice which were needle inoculated with spirochetes (Table 11). This activity lasted the entire duration of the study. In contrast, no such activity was detected in sera collected from the group of mice infected by ticks or the control groups at any time interval.

Table 11. *In vitro* spirochete neutralization activity of sera collected at different intervals from BALB/c mice infected with *B. burgdorferi* either by needle inoculation or by tick transmission

Days Post Infection	Clean ticks	PBS injected	Needle inoc. Infec.	Tick trans. infec.
3	1,753 ± 19	1,616 ± 171	312 ± 1	1,464 ± 52
7	-	-	424 ± 30	1,979 ± 85
10	-	-	388 ± 5	1,654 ± 47
14	-	-	789 ± 29	2,041 ± 140
21	-	-	603 ± 18	1,353 ± 88
28	1,403 ± 101	1,391 ± 121	413 ±15	1,408 ± 40
56	-	-	359 ± 42	1,862 ± 265
84	1,686 ± 167	1,595 ± 93	408 ± 39	1,457 ± 47

1 X 10^5 spirochetes were incubated with 1:20 diluted sera from different groups in the presence of guinea pig complement in a total of 200 µl BSK II. Six hrs later, one µCi of ^{3}H adenine (20 µl of BSK II) was added along with 600 µl of fresh medium, and incubated further for 96 hrs. Amount of label incorporated was determined and all values are presented in CPM ± S.E.

CHAPTER IV

DISCUSSION

The biological transmission of any kind of pathogen by an arthropod vector involves complex interactions at the host-vector-pathogen interface (Wikel, 1996a). The adaptations made by all the players for their survival are quite elegant and extremely intricate. Hematophagous arthropods such as ticks have developed antihemostatic, anti-inflammatory, and immunomodulatory properties in their saliva for successful acquisition of a blood meal. (Ribeiro *et al.*, 1985). The process of blood feeding makes use of a large number of redundant mechanisms to out wit the host homeostatic machinery. Saliva of hematophagous insects interferes with the host clotting mechanism at a variety of steps in the intrinsic and extrinsic mechanism of clotting (Champagne and Valenzuela, 1996). Similarly, diverse anti-inflammatory and immune mediators are downregulated by blood feeding arthropods during feeding (Wikel, 1996a).

The vector borne pathogens have exploited such adaptations on the part of the vector arthropod for their own transmission, establishment, and survival in the vertebrate host. The adaptations made by pathogens are equally remarkable in terms of their ability to cope with two completely different and potentially hostile environments i.e. homeothermal vertebrate host and a poikilothermal arthropod vector. It is likely that a large number of changes are necessary in their metabolic profile for their survival during transmission from vector to host. Thus, it is logical to conclude that the metabolic and morphological (surface coat proteins) make up of a pathogen while in the vector might be quite different from that in the vertebrate host. In addition, isolation of a pathogen either

from a vertebrate host or arthropod vector, and subsequent prolonged cultivation *in vitro* in enriched media can result in changes in the protein profile of the pathogen (Ramachandra *et al.*, 1994).

Lyme borreliosis is a multisystem disorder involving predominantly the skin, the nervous system, and joints (Steere, 1989). It is caused by spirochete *B. burgdorferi* which is transmitted by ixodid ticks of the *I. ricinus* complex (Burgdorfer *et al.*, 1982). This organism has been isolated from a variety of vertebrate hosts and the vector ticks (Nocton and Steere, 1995). This organism can be cultured *in vitro* continuously in a complex and highly enriched medium called BSK II (Barbour, 1984).

A large number of initial studies conducted to understand various laboratory and clinical aspects, pathogenesis, and for development of prophylaxis against this malady were performed by needle inoculation of such culture grown spirochetes in different experimental models (reviewed by Sigal, 1997). This approach has the obvious flaw that it completely leaves out the role of vector tick in terms of its effects on host homeostasis at the time of pathogen transmission. The down regulation of host hemostatic, inflammatory and immune mechanisms especially at the tick bite site might create a compromised microenvironment which can have profound effect on subsequent spirochete establishment and the nature of host immune responses mounted against it.

The immune response mounted by the vertebrate host against the needle inoculated and tick transmitted *B. burgdorferi* differs (Roehrig *et al.*, 1992). The role of the tick vector in the modulation of the host immune response is beginning to be realized (Wikel *et al.*, 1996a and b). The adaptations made by the spirochete in terms of selective expression of certain proteins in the vertebrate host as compared to that with in the tick

are becoming known (Schwan *et al.*, 1995). Several studies have identified spirochetal proteins that react with sera from infected patients or animals but are not expressed by cultured spirochetes (Champion *et al.*, 1994; Akins *et al.*, 1995; Suk *et al.*, 1995; Wallich *et al.*, 1995; Seiler and Weis, 1996).

The purpose of the present study was to determine the differences in the humoral and cellular immune responses mounted by BALB/c mice against tick transmitted and needle inoculated infection with *B. burgdorferi* strain B31 at different time intervals during the course of infection. BALB/c mice were selected for this study because they can be infected experimentally resulting in development of mild lesions and symptoms which resemble human Lyme borreliosis (Barthold *et al.*, 1990; Ramachandra *et al.*, 1993). Low passage *B. burgdorferi* strain B31 was used in this study to infect *I. scapularis* nymphs and BALB/c mice. The presence of a large amount of OspA in the whole spirochete extract prepared from this strain interfered with preparative SDS-PAGE fractionation using Prep Cell Model 491 (Bio-Rad). OspA contaminated the entire spectrum of fractions obtained . Therefore, a mutant strain of B31 which lacked the expression of OspA and B was used for this purpose.

A 100 % infection rate in *I. scapularis* nymphs with *B. burgdorferi* was obtained by feeding unfed larvae on infected BALB/c mice. An infection rate of $\geq$ 80 % has been reported by Piesman (1993) using the same technique to rear infected *I. scapularis* nymphs. Wikel *et al.* (1997) reported an infection rate of 100 % in *I. scapularis* nymphs reared in the same manner. These nymphs successfully transmitted infection to BALB/c mice on subsequent feeding as evidenced by reisolation of the spirochete from the urinary bladder of all such mice. Wikel *et al.*, (1997) reported that 100 % naive mice (no previous

exposure to ticks) when infested with six infected unfed *I. scapularis* developed *B. burgdorferi* infection as evidenced by isolation of the spirochete from the ear punch biopsies.

Similarly, successful infection of BALB/c mice by intraperitoneal inoculation of spirochetes was achieved during this study. Spirochetes could be reisolated from urinary bladders of all the mice inoculated with spirochetes intraperitoneally. Similar results were reported by Ramachandra *et al.*, (1993) who used 1X 10^7 spirochetes to inoculate BALB/c mice by intraperitoneal route. Establishment of Lyme borreliosis by intraperitoneal inoculation of 1 X 10^8 spirochetes has also been reported by Benach *et al.*, (1988).

Spirochetes could be consistently isolated as early as seven days post infection from the urinary bladders of all the mice infected with Lyme borreliosis either by needle inoculation or by tick transmission. Schwan *et al.* (1988) have reported the urinary bladder of mice to be a consistent source of spirochetes in infected mice. de Souza *et al.*, (1993b) could also isolate spirochetes from the urinary bladders of C3H mice at four days post infection following intradermal inoculation. Ramachandra *et al.*, (1993) reisolated spirochetes from urinary bladders of BALB/c mice at four weeks after needle inoculation of spirochetes.

From 20 mice infested with *B. burgdorferi* infected *I. scapularis* nymphs (six nymphs per mouse, 120 total), 82 (68.33 %) were recovered after feeding to repletion. Wikel *et al.*, (1997) reported a recovery of 65 % following infestation of naive mice with similar number of nymphal *I. scapularis* ticks. For pathogen free ticks, 66 replete nymphs (78.57 %) were recovered from 14 mice (six nymphs per mouse, 84 total) in the present

study. Wikel *et al.*, (1997) recorded a slightly lower recovery rate of 55.60 % for pathogen free ticks infested on BALB/c mice.

Use of Prep Cell model 491(Bio-Rad) allowed an excellent fractionation of a complex mixture of proteins present in the whole spirochete extract of OspA and B negative mutant of *B. burgdorferi* strain B31. The usefulness of this technique has previously been demonstrated. Brown *et al.*, (1995) fractionated merozoite membranes and whole merozoite antigens of *Babesia bovis* using 10 and 15 % acrylamide concentration in the fractionation resolving gel. Subsequently, they used these fractions for the tentative identification of relevant molecules that stimulate proliferation of T-helper cell lines and clones derived from cattle immune to challenge infection with *B. bovis*.

Since, SDS-PAGE fractionation is done under reducing conditions, conformational epitopes on different polypeptides will be likely destroyed. However, because of the reducing conditions, both soluble and membrane bound proteins can be targeted with this technique (Brown *et al.*, 1995). Bergman *et al.*, (1995) used the same technique to fractionate salivary gland extract from *Dermacentor andersoni* using 5, 7.5, or 10 % acrylamide in the resolving gel. Various proteins present in these fractions were tested for their effects on Con A-induced blastogenesis of normal murine splenocytes.

In the present study, seven different fraction pools with diverse protein constitution were obtained by pooling fractions obtained from three different prep cell runs using 7.5, 10, and 12% acrylamide in the resolving gel. The fractionation with 7.5 % acrylamide concentration gave best resolution of polypeptides in the molecular weight range of 45-56 kDa. Similarly, respective best ranges for 10 and 12 % acrylamide were

10-33 kDa and 10-20 kDa in the present study. Brown *et al.*, (1995) using 10 % acrylamide concentration were able to obtain good resolution in the molecular weight range of 20-95 kDa. With the 15 % acrylamide gel, they obtained a step-wise progression of bands ranging from 14-25 kDa. Bergman *et al.*, (1995) used acrylamide concentrations ranging from 5 to 10 % to obtain fractions containing proteins in the molecular weight range of approximately 12 to 230 kDa. The apparent differences in the best resolution range could be due to the initial amount of total protein loaded on the gel as well as the length of resolving gel.

The fractions were pooled only with fractions from the same fractionation run. The fractions with similar protein profiles were pooled in order to obtain enough quantity that could be used in splenocyte proliferation assays conducted at five different time intervals post infection. The purpose of testing these fraction pools was to identify any polypeptides that induced a differential proliferative response in splenocytes from mice with tick transmitted infection and needle inoculated infection.

Whole spirochete extracts obtained from both the strains of spirochete, all fraction pools, and recombinant OspA (31 kDa) were tested for their effects on *in vitro* proliferative responses of splenocytes obtained from BALB/c mice infected with *B. burgdorferi* by either tick transmission or needle inoculation. Splenocytes obtained from groups of mice infested with clean nymphs and injected with sterile PBS subjected to similar stimulations were used as matched controls for tick transmitted and needle inoculated infection, respectively. In addition to all the spirochetal antigens, effects of Con A (T-cell mitogen) and LPS (B-cell mitogen) on proliferative responses of splenocytes from all the groups were also tested. The splenocytes were collected at week

one, two, four, eight and 12 post infection and were subjected to a three day and five day lymphocyte proliferation assay.

In three day culture assay, the Con A responsiveness of splenocytes from mice with tick transmitted infection was impaired as compared to the splenocytes from mice with needle inoculated infection at all the interval tested except at one week post infection. The splenocytes from mice infected by tick transmission of *B. burgdorferi* showed reduced proliferation in response to Con A as compared to their matched control of splenocytes from mice infested with pathogen free ticks also. Con A response of lymphocytes from human patients with active Lyme disease was reduced as compared to healthy controls (Dattwyler, 1986). Benach *et al.*, (1988) have reported reduced proliferative responses to Con A by splenocytes from mice with Lyme borreliosis as compared to normal mice suggesting a period of decreased mononuclear cell function. Both C3H and BALB/c mice infected for seven and 14 days had diminished responses to Con A and LPS relative to those of controls (de Souza *et al.*, 1993a). de Souza *et al.*, (1993b) also found that lymphocytes derived from the spleens of C3H mice infected with needle inoculation of spirochetes in the shoulder at seven and 14 days post-infection showed impaired proliferative responses to both Con A and LPS.

The Con A response of needle inoculated infection mice was much lower than that of the tick transmitted infection group in the present study at one week post infection (three day assay). The larger number of spirochetes (1×10^7) given by needle might have caused overwhelming infection resulting in reduced responsiveness of the splenocytes to Con A. In the tick transmitted infection, a single tick injects only about 10^3 to 10^4 spirochetes during feeding to repletion (Burgdorfer *et al.*, 1982). The Con A response of

splenocytes from mice with needle inoculated infection was lower than the matched control (PBS injected) one week, and almost the same at 12 weeks post infection but showed hyper-responsiveness at two and eight weeks post infection. It is possible, that following the initial period of overwhelming infection, the splenocytes recovered and proliferated vigorously in response to Con A. Hyper-responsiveness of lymph node cells obtained from mice with inoculation of spirochetes in the foot pad to LPS has previously been reported (de Souza *et al.*, 1992). Responses to Con A were not tested in this study.

Splenocytes from both the control groups showed equally significant (SI>4) proliferation in response to Con A at all the intervals except at 12 weeks where the splenocytes from mice given pathogen-free tick infestation showed higher responses as compared to PBS injected mice splenocytes. Tick infestation has been shown to reduce responsiveness of lymphocytes to mitogens. Infestation of guinea pigs with tick *Dermacentor andersoni* reduced the *in vitro* responsiveness of lymphocytes to T-cells mitogen Con A (Wikel, 1982). Salivary gland extracts prepared from female *D. andersoni* suppressed normal murine lymphocyte responsiveness to Con A *in vitro*. Wikel *et al.*, (1997) observed that repeated infestation with *I. scapularis* nymphs did not induce any acquired resistance to subsequent feeding. However, following repeated infection with pathogen-free nymphs, there was a definite interference with transmission of *B. burgdorferi* by infected ticks. This indicates that, upon repeated infestation, mice acquired the ability to neutralize certain factors in tick saliva that help in establishment of infection. These factors can be immunosuppressive but may be acting locally and do not affect the responsiveness of splenocytes to mitogens in case of *I. scapularis*.

In the five day assay, the similar trend of Con A responsiveness by different groups was maintained in general but the SI's were numerically smaller in most cases. The five day assay was performed since the conventional antigen-specific responses, involving very small percentages of cells generally require five days for maximal expression (Colligan *et al.*, 1991). The mitogenic responses involving entire populations of cells are maximal in two to three days, with the decline during longer incubation being due to depletion of nutrients. Thus, higher SI's at an incubation period of 72 hours point towards the nonspecific mitogenic effects of Con A. Surprisingly, the numerical values of SI's for Con A responsiveness were higher in the five day assay as compared to those in the three day assay except in the case of mice with uninfected tick infestation.

The LPS induced proliferative responses of splenocytes from mice with tick transmitted infection, when compared with those of splenocytes from needle inoculated infection, were variable. The SI's were smaller as compared to those for stimulation with Con A, which can be attributed to the presence of smaller number of B-cells as compared to T-cells among the splenocytes. The LPS response of the tick transmitted infection group splenocytes was higher at one week, lower at two weeks, similar at four weeks, much lower at eight weeks and once again similar at 12 weeks post infection as compared to splenocytes obtained from needle inoculated group. The LPS induced proliferation in splenocytes from mice with tick transmitted infection was diminished than their matched control of splenocytes from mice given an infestation with pathogen free ticks except at one week post-infection where almost the same level of responsiveness was noted. The LPS response of splenocytes from mice with needle inoculated infection was lower at one week, up to same level at two weeks, much higher at eight weeks and almost similar at 12

weeks post-infection as compared to their matched control i.e. PBS injected group. The hyper-responsiveness observed at eight weeks was also observed for Con A stimulation at this interval. de Souza *et al.*, (1993b) have reported such enhanced responses to LPS in the lymph node cells obtained from mice inoculated with spirochetes in the foot pad but not to Con A. The impaired LPS responsiveness of splenocytes from mice administered infection by needle in the shoulder has also been reported (de Souza *et al.*, 1993b). They did not test for LPS responses of splenocytes from mice with tick transmitted infection.

In the five day assay numerically smaller SI's were observed for all groups. In fact, the SI's for tick transmitted and needle inoculated infection groups dropped below significant level i.e. less than four at certain intervals. Only at 12 weeks post-infection, five day assay, the SI's were numerically slightly larger than those at three day assay.

In the present study, the whole spirochete extracts obtained from both type strain B31 and OspA and B deficient strain of *B. burgdorferi* caused significant proliferation of splenocytes from both infected (needle or tick) and control groups. This observation reflects the presence of nonspecific mitogens in the spirochete extracts which has previously been reported in the literature. Lymphocytes from uninfected healthy human subjects, serving as controls for patient studies, were found to proliferate when incubated with *B. burgdorferi* antigen (Zoschke *et al.*, 1991). de Souza *et al.*, (1992) showed that *B. burgdorferi* antigens induced nonspecific proliferative responses in B-cell enriched fraction of naive murine splenocytes. This effect was dissimilar to that of LPS because splenocytes from C3H/HeJ mice showed proliferation although this strain of mice is an LPS non-responder. Schoenfeld *et al.*, (1992) demonstrated a B-lymphocyte mitogen produced by *B. burgdorferi* which had potent mitogenic activity when cultured with

lymphocytes from naive C57BL/6, C3H/HeJ, or BALB/c mice. Tai *et al.*, (1994) have reported that normal human B-cells and not T-cells proliferated when incubated with sonicated *B. burgdorferi*. Antigen specific proliferative responses to borrelial antigens have also been described. Yoshinari *et al.*, (1991) have described the proliferation of T-cells obtained from Lyme disease patients in response to different *B. burgdorferi* antigens. Dattwyler *et al.*, (1986) did not see any proliferation in lymphocytes from normal human subjects in response to *B. burgdorferi* antigens whereas proliferative cellular response in patients with Lyme disease were consistently present. de Souza *et al.*, (1993a) observed that at seven days post-infection (needle inoculation), responses of splenocytes from C3H mice to *B. burgdorferi* as stimulus were decreased. Elevated responses to borrelial antigens were observed only at 26 weeks and later, post-infection in C3H mice. In BALB/c splenocytes, elevated responses were not seen, except at two and 52 weeks post-infection, but proliferation at those times was only marginally higher for splenocytes from infected mice than for those from control mice (de Souza *et al.*, 1993a).

In the present study, the proliferative response of splenocytes from mice with tick transmitted infection to WSE (B31) were lower than that of splenocytes from mice with needle inoculated infection at all intervals post-infection, except at one week post-infection where they were at equivalent levels. When compared to matched controls, the proliferative responses of splenocytes from mice with tick transmitted infection had diminished responses to WSE (B31) as stimulant at all intervals post-infection. Whereas responses of needle inoculated infection mice to WSE (B31) were variable being lower at one week, slightly higher at two weeks, much higher at eight weeks, and equivalent at 12 weeks post-infection as compared to their matched control. The results of five day assay

were similar with SI's falling below the cutoff value of four, indicating reduced viability of cells.

Ma and Weis (1993) showed that the *B. burgdorferi* lipoproteins OspA and OspB could cause naive murine B cells to proliferate *in vitro* and have cytokine-stimulatory properties. Tai *et al.*, (1994) showed similar effects of *B. burgdorferi* antigens on normal human B lymphocytes and mononuclear cells. However, our results show that whole spirochete extract from B31 strain that lacked OspA and B expression also induced significant proliferation of splenocytes from both infected and control mice indicating presence of non-specific mitogenic properties. Difference in proliferative responses of lymphocytes from mice with tick transmitted infection versus needle inoculated infection to WSE (AB def.) was quite apparent. At all intervals tested tick transmitted infection lymphocytes proliferated at lower level than the needle inoculated infection lymphocytes, except at one week post infection where the responses were equivalent. As compared to the matched controls, lymphocytes from mice with tick transmitted infection had diminished responses whereas those from needle inoculated infection had elevated responses. The results of five day assay were similar except for much smaller SI values.

Our results strongly indicate the presence of antigens other than OspA and B having nonspecific mitogenic effects on murine splenocytes. In fact, fraction pool seven, obtained by SDS-PAGE fractionation of WSE (AB def.), which contained a 20 kDa borrelial protein showed non-specific mitogenic effects on splenocytes from both *B. burgdorferi* infected and control group. The proliferative responses to fraction pool seven were lower in tick transmitted infection group than those of needle inoculated infection group at all intervals, except at one week post-infection where the opposite was true. An

overwhelming number of spirochetes, causing generalized immunosuppression might have been the reason for this observation. When compared to its matched control, proliferative responses of the tick transmitted group to fraction pool seven were variable being higher at one week, lower at two weeks, higher at eight weeks and lower again (in fact, SI<4) at 12 weeks post infection. In contrast, proliferative responses of splenocytes from needle inoculated infection group to fraction pool seven were consistently elevated as compared to the matched control of PBS injected mice except at one week post-infection. The results followed the same trend in the five day assay also.

The possible existence of mitogenic lipoproteins other than OspA and B produced by *B. burgdorferi* has been discussed by Tai *et al.*, (1994). A major low-molecular-weight 10 kDa-M_r protein purified and characterized by Katona *et al.*, (1992) is one such possibility. The recombinant OspA tested for it's stimulatory effects on splenocytes from both naive and infected mice in the present study was non-lipidated. In keeping with the previous findings (Weis *et al.*, 1994), it lacked any mitogenic effects.

The borrelial antigens present in fraction pool one through six, respectively containing 30.5-38.5 kDa, 45 kDa, 50 and 51 kDa, 56 kDa, 27 kDa, and 33 kDa borrelial proteins did not cause significant level of proliferation in splenocytes from mice with tick transmitted infection or in both the control groups. However, at four weeks post-infection (three day assay) splenocytes from the needle inoculated infection group showed barely significant proliferation in response to fraction pool one (30.5-38.5 kDa) and fraction pool three (50 and 51 kDa) with respective SI's of 4.08 and 4.35.

Antigen-specific responses to borrelial antigens have been difficult to study using unfractionated cell populations because it has led to variable and conflicting conclusions

(de Souza *et al.*, 1993a). One reason for this is the non-specific mitogenic effects of *B. burgdorferi* antigens on even naive lymphoid cell populations. As discussed above, amount of initial spirochete inoculum, route of inoculation, site of inoculation, and the source of lymphoid cell populations complicate the interpretation of results

In the present study, an attempt was made to study the differences in antibody response to Lyme borreliosis initiated by tick transmission versus needle inoculation. Sera collected at three, seven, ten, 14, 21, 28, 56, and 84 days post infection were monitored for the presence of spirochete specific IgM and IgG by a sensitive ELISA protocol, and their specific reactivity with borrelial antigens was determined by Western immunoblot using whole spirochete extract from *B. burgdorferi* B31 strain. Distinct differences in time of appearance, magnitude and specificity (determined by Western immunoblot) of anti-spirochete IgM and IgG antibody were found in tick transmitted versus needle inoculated infection with *B. burgdorferi*. In the sera from needle inoculated mice, the anti-spirochete IgM antibody response was apparent as early as the third day post-infection, peaked at day seven and then an additional peak was observed at day 21 and 28. On Western immunoblot analysis, the reactivity was against 39 and 24 kDa antigen which lasted till 21 days post-infection. On day 28, reactivity to only the 39 kDa protein was observed which disappeared at 56 and 84 days post-infection. In contrast, sera from mice with tick transmitted infection did show same level of absorbance on day three post-infection but no anti-spirochete IgM reactivity was observed on Western blot analysis until day 10 post-infection and that too only against 39 kDa protein. This reactivity lasted only until day 21 post-infection. Overall, the magnitude of anti-

spirochete IgM was higher in needle inoculated infection as compared to tick transmitted infection.

Ramachandra *et al.*, (1993) using needle inoculation methods of infection in BALB/c mice found a similar trend of IgM response with spirochete specific IgM antibodies appearing as early as three days post-infection, peaked on days eight and nine, and gradually declined after day 12. Immunoblotting analysis for the specificity of anti-spirochete IgM antibody was not done in this study. de Souza *et al.*, (1993b) using C3H mice showed that the route of inoculation of spirochetes cause variation in the antibody response. Shoulder- but not foot-inoculated mice developed IgM reactivity to *B. burgdorferi* on day seven. However, they did not study the kinetics of antibody response following tick-transmitted infection. Schwan *et al.* (1989) studied the antibody response of white-footed mouse, *Peromyscus leucopus*, following needle inoculation of 2×10^8 spirochetes by intraperitoneal route. They detected anti-spirochete IgM antibody in circulation within one or two days after inoculation, with a peak at four or five days post inoculation. A secondary peak in IgM was observed on day 21 post-inoculation. These results are in agreement with our findings.

Differences in spirochete specific IgG antibody elaboration by mice receiving needle inoculated and tick-transmitted infection were also seen. Anti-spirochete IgG antibody was detected on third day post infection and there after showed a gradual increase through out the duration of experiment with maximum absorbance value on day 84 in the case of needle inoculated infection. However, specific reactivity, by immunoblotting, to borrelial antigens was not observed until day 10 post-infection when the serum reacted with a 39 kDa and a 31 kDa (OspA) protein. Subsequently, this

reactivity was maintained throughout the course of this study. In contrast, absorbance values not more than those for control sera were observed in the sera from mice infected by tick transmission till day 56 when significant levels of IgG were detected. On day 84 post infection, about the same amount of IgG was again detected but level was still much lower than that in case of needle inoculated infection. The only reactivity in the sera from mice given tick transmitted infection was to the 39 kDa protein which was observed 56 days post-infection and also at 84 days post infection.

Schwan *et al.*, (1989) found that white-footed mouse, *P. leucopus* began circulating anti-*B. burgdorferi* antibodies as early as one or two days post inoculation and showed a continuous increase in circulating antibody out to day 84. On immunoblot analysis, the sera showed reactivity to a large number of spirochetal antigens at different times post-infection. Ramachandra *et al.*, (1993) detected specific IgG in sera of mice needle inoculated with spirochetes at days seven and eight post infection. They observed a constant increase in titer throughout the eight week duration of the experiment. By immunoblotting, they observed that the BALB/c sera reacted only with one polypeptide band of approximately 39 kDa starting at 14 days post-infection and persisting throughout the course of study. Barthold *et al.*, (1990) showed that sera from mice infected by needle inoculation reacted with 20 kDa, 31-32 kDa (OspA), 36 kDa (OspB) and 41 kDa (flagellin). de Souza *et al.*, (1993b) showed IgG seroconversion among shoulder inoculated C3H mice on day 10 and among foot inoculated mice on day 14. The minor differences in these results can be attributed to the strain of spirochetes and mice used. None of the above mentioned studies used tick transmission method of spirochete to study the antibody response kinetics.

The lower levels and later appearance of antibodies observed during tick transmitted infection in the present study could be because of small inoculum of infection injected by the ticks as compared to the needle inoculation. It is estimated that 10^3 to 10^4 organisms are transmitted by each tick (Burgdorfer *et al.*, 1982). In addition the immunosuppressive properties of tick saliva may interfere with the proper presentation of antigen and subsequent development of immune response. An anti-31 kDa (OspA) antibody was detected in sera from mice with needle inoculated infection and appeared as early as ten days post infection. The sera from mice with tick transmitted infection lacked anti-OspA response at all times. These results are in accordance with those of Roehrig *et al.*, (1992) who did not find any anti-OspA and -OspB response in hamsters infected by tick transmission of the organism. In human patients also, an anti-OspA or -OspB response is not detected till much later in the disease course if at all (Steere, 1989).

Several explanations for the lack of anti-OspA response in natural (tick transmitted) Lyme borreliosis have been put forward. Small inoculum of spirochetes given intradermally mimics the natural infection and no anti-OspA response is seen in such animals (Barthold *et al.*, 1995). More recently, surface antigen modulation by the spirochetes has been determined during tick transmission. Schwan *et al.*, (1995) demonstrated a decrease in expression of OspA and increase in expression of OspC on *B. burgdorferi* during acquisition of blood meal by the tick. This change was not due only to temperature change but also due to other environmental changes associated with blood meal. Further, de Silva *et al.*, (1996) using fluorescent immunostaining showed that the spirochetes in fed ticks lacked OspA expression whereas it was present in unfed ticks.

In the present study, spirochetes could be reisolated from the urinary bladder of mice infected by either tick transmission or needle inoculation of spirochete at all time intervals. The persistence of spirochetes in the face of humoral immune response in immunocompetent animals has been described earlier also (Seiler and Weis, 1996). Existence of *B. burgdorferi* in an immunologically privileged site, surface antigenic modulation and debatable hypothesis of intracellular localization of spirochetes are some of the explanations put forward to explain this phenomenon (Seiler and Weis, 1996).

The role of anti-*B. burgdorferi* antibodies in providing protection against Lyme borreliosis is well documented (reviewed by Simon *et al.* 1991b; Nocton and Steere, 1995; and Seiler and Weis, 1996). High levels of borreliacidal antibodies develop in response to needle inoculation of hamsters with Lyme disease spirochete (Schmitz *et al.* 1991; Lovrich *et al.* 1991). In the present study, the development of borreliacidal antibodies in the sera from mice with tick transmitted and needle inoculated infection was monitored using an *in vitro* spirochete neutralization assay using complement mediated killing. Borreliacidal antibodies were present in sera from needle inoculated mice at all the time intervals tested appearing as early as three days post-infection. In contrast, the sera from mice with tick transmitted infection did not have any borreliacidal activity at any time.

BALB/c mice suffer a mild form of Lyme borreliosis which is accompanied by a relatively poor antibody response to *B. burgdorferi* as compared to more susceptible mice such as C3H mice (Barthold *et al.* 1990; Ramachandra *et al.* 1993). Further, tick transmitted infection group had lower amount of anti-spirochete antibody than the mice with needle inoculated infection group in the present study. Finally, lack of anti-OspA

response in tick transmitted infection may be another reason for lack of borreliacidal activity in the sera from such mice.

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APPENDICES

APPENDIX I

Reagent Compositions

Section Page

Section 1: Buffers and Media Compositions

Phosphate Buffered Saline (PBS, 0.15 M, pH 7.2)

1. Dissolve the following in 900 ml of triple distilled water.

Potassium phosphate (monobasic, KH_2PO_4))	2.45 g
Sodium Phosphate (dibasic, Na_2HPO_4)	8.10 g
Sodium Chloride (NaCl)	4.38 g

2. Bring the final volume to one liter. The pH does not need to be adjusted.

Barbour-Stoenner-Kelly II Medium (BSK II)

1. Dissolve 100 ml of 10 X CMRL (Gibco, Grand Island, NY) in 900 ml of triple distilled water to prepare 1 X solution.
2. Dissolve the following constituent in order in 1 X CMRL 1066.

Neopeptone	5 g
BSA Fr. V	50 g
Yeastolate	2 g
HEPES	6 g
Glucose	5 g
Sodium Citrate	0.7 g
Sodium Pyruvate	0.8 g
N-Acetylglucosamine	0.4 g
Sodium Bicarbonate	2.2 g

3. Adjust pH to 7.6 with 1N NaOH
4. Filter sterilize using 0.22 µm nitrocellulose membrane sterivex filter unit with bell fitting (Millipore, Bedford, MA)
5. Prepare 200 ml of 7 % Gelatin in distilled water and autoclave at 121 °C for 15 minutes. Let it cool down to room temperature and add to the medium under sterile conditions.
6. Add unheated rabbit serum (Gibco, Grand Island, NY) to 6 %.
7. Incubate 10 ml of this medium at 37 °C overnight for sterility check.
8. Store the medium in one liter media bottles (Corning, New York, NY) under refrigerator.

Phosphate Citrate Buffer (pH 5.0)

1. Solution A: 0.1 M Citric acid. Dissolve 10.5 g Citric acid (monohydrate) in 500 ml distilled water.
2. Solution B: 0.2 M Sodium phosphate (dibasic). Dissolve 14.2 g Sodium phosphate dibasic) in 500 ml distilled water.
3. Mix 48.5 ml of solution A with 51.5 ml of solution B. pH should be around 5.0. Adjust if necessary.

Tris-Saline Buffer (10 mM Tris-HCl, 0.9 % NaCl, pH 7.4)

1. Dissolve the following in one liter of distilled water.
   Tris base 2.42 g
   NaCl 18.0 g
2. Adjust to pH 7.4 with 1 N HCl, and adjust the final volume to two liters.

RPMI 1640 Medium

1. Dissolve one packet of RPMI 1640 medium powder (Gibco, Grand Island, NY) in 900 ml of triple distilled water in a 2000 ml flask. Place a Teflon coated stir bar at the bottom of flask and place it on a magnetic stirrer.
2. Add 2.0 g of sodium bicarbonate.
3. Dissolve one vial of 200 mM L-Glutamine (Gibco, Grand Island, NY) in 20 ml of MEM-non essential amino acid solution (10 mM, 100 X) (Gibco, Grand Island, NY) and add 10 ml of this solution to the medium.
4. Add 10 ml of MEM vitamin solution (100 X) (Gibco, Grand Island, NY).
5. Dissolve all the components by with gentle stirring for about 2 hrs.
6. Adjust pH to 7.1 with 1N HCl.
7. Bring the total volume to one liter.
8. Filter sterilize using 0.22 μm filter with bell fitting (Millipore, Bedford, MA)
9. Mix 87.75 ml of the above medium with 10 ml of heat inactivated (56 °C for 30 minutes) fetal calf serum (Gibco, Grand Island, NY), 2 ml of Penicillin/Streptomycin solution (Penicillin 5000 units, Streptomycin 5000 μg per ml) (Gibco, Grand Island, NY) and 0.25 ml of Gentamicin solution (10 mg/ml) (Gibco, Grand Island, NY) to get the final working medium.

Section 2: Electrophoresis Reagents and Recipes

Resolving Gel Buffer stock (1.5 M Tris-HCl, pH 8.8)

1. Dissolve 27.23 g Tris base in approximately 80.0 ml distilled water.
2. Adjust pH to 8.8 with 6 N HCl. Make to 150.0 ml with distilled water and store at 4 °C.

Stacking Gel Buffer Stock (0.5 M Tris-HCl, pH 6.8)

1. Dissolve 6.0 g Tris base in approximately 60 ml distilled water.
2. Adjust to pH 6.8 with 6 N HCl. Make to 100.0 ml with distilled water and store at 4 °C.

Polyacrylamide Gel Recipes:

Resolving Gel (Preparative and Analytical SDS-PAGE)

	Percent Acrylamide Concentration		
	7.5	10	12
Resolving gel stock	20.0 ml	20.0ml	20.0 ml
Deionized water	39.8 ml	33.1 ml	27.8 ml
Acrylamide/bis stock (30% T/2.67% C)	20.0 ml	26.7 ml	32.0 ml
10% Ammonium persulphate	0.2 ml	0.2 ml	0.2 ml
TEMED	0.02 ml	0.02 ml	0.02 ml

Stacking gel, Four percent (Preparative and Analytical SDS-PAGE)

	Acrylamide conc.
	4.0
Stacking gel stock	20.0 ml
Deionized water	39.8 ml
Acrylamide/bis stock (30% T/2.67% C)	20.0 ml
10% Ammonium persulphate	0.2 ml
TEMED	0.02 ml

10 X electrode/tank buffer (25 mM Tris, 192 mM Glycine, pH 8.3)

1. Dissolve the following in 900 ml of distilled water.
   Tris base 30.3 g
   Glycine 144.0 g
2. Adjust the volume to one liter.
3. To make one liter SDS-PAGE electrode buffer, dissolve 1 g SDS in 100 ml of distilled water, add 100 ml of 10 X electrode/tank buffer and bring the volume up to one liter. Keep chilled in the refrigerator.

3 X SDS PAGE sample buffer

1. Dissolve 2.28 g tris base in 40 ml distilled water.
2. Adjust pH to 6.8 with 10N HCl.
3. Dissolve 5.0 g SDS in the above.
4. Add 30.0 ml glycerol.
5. Add 3.0 ml of 0.05 % bromophenol blue.
6. Bring the volume up to 85.0 ml with distilled water.
7. Add 150 µl of $-mercaptoethanol to 850 µl of the above solution.

APPENDIX II

In vitro splenocyte proliferation assay data presented as actual CPM ± S.E obtained in response to different mitogens and antigens at different intervals following infection of BALB/c mice by tick transmission or needle inoculation of *Borrelia burgdorferi*.

Table A. *In vitro* proliferative responses of splenocytes from BALB/c mice infected with *B. burgdorferi*, either by tick transmission or by needle inoculation, to Concanavalin A (Con A), Lipopolysaccharide (LPS), whole spirochete extract from type strain B31 (WSE B31), whole spirochete extract from OspA and B negative mutant (WSE AB def.), fraction pools from preparative SDS-PAGE of WSE (AB def., Pool 1 through 7), and recombinant OspA (Rec. OspA). One week post infection, Three day assay

Treatment	Tick trans. inf.	Needle inoc. inf.	Uninfected ticks	PBS inject.
Con A	20,238 ± 392	15,091± 364	37,522 ± 731	24,521 ± 543
LPS	3,616 ± 24	3,255 ±35	4,928 ± 177	3,371 ± 131
Cells Alone	452 ± 24	577 ± 15	688 ± 37	443 ± 12
WSE (B31)	4,864 ± 102	5,553 ± 181	8,480 ± 235	5,566 ± 169
WSE (AB def.)	5,050 ± 303	6,169 ± 74	9,088 ± 187	4,892 ± 72
Pool 1	1,227 ± 85	1,038 ± 93	997 ± 31	1,170 ± 21
Pool 2	416 ± 32	524 ± 38	465 ± 1	529 ± 11
Pool 3	1,264 ± 43	909 ± 22	1,271 ± 63	1,341 ± 136
Pool 4	421 ± 36	560 ± 14	502 ± 33	623 ± 25
Pool 5	645 ± 128	469 ± 19	459 ± 15	586 ± 5
Pool 6	387 ±25	483 ± 19	428 ± 5	528 ± 24
Pool 7	6,192 ± 305	2,658 ± 116	4,597 ± 240	5,069 ± 342
Rec. OspA	772 ± 117	1,191± 58	909 ± 64	927 ± 57

5×10^5 splenocytes were incubated with respective antigen/mitogen or without stimulation (cells alone) in triplicate for 54 hrs and then pulsed with one μCi of ^{3}H-thymidine per well. Data is presented as mean CPM ± S.E obtained after harvesting the cells 18 hrs later and counting in liquid scintillation counter.

Splenocytes from mice infested with pathogen free ticks or injected with PBS served as matched controls for tick transmitted and needle inoculated infection, respectively.

Table B. *In vitro* proliferative responses of splenocytes from BALB/c mice infected with *B. burgdorferi*, either by tick transmission or by needle inoculation, to Concanavalin A (Con A), Lipopolysaccharide (LPS), whole spirochete extract from type strain B31 (WSE B31), whole spirochete extract from OspA and B negative mutant (WSE AB def.), fraction pools from preparative SDS-PAGE of WSE (AB def., Pool 1 through 7), and recombinant OspA (Rec. OspA). One week post infection, Five day assay

Treatment	Tick trans. inf.	Needle inoc. inf.	Uninfected ticks	PBS inject.
Con A	29,230 ± 426	16,536± 556	30,867 ± 47	32,804 ± 207
LPS	3,223 ± 164	2,109 ± 126	1,999 ± 60	2,821 ± 137
Cells Alone	687 ± 19	714 ± 553	685 ± 33	692 ± 21
WSE (B31)	2,055 ± 3	2,821 ± 214	2,344 ± 178	1,510 ± 34
WSE (AB def.)	2,097 ± 82	2,881 ± 134	2,221 ± 107	2,016 ± 23
Pool 1	856 ± 39	833 ± 88	560 ± 30	828 ± 51
Pool 2	830 ± 37	966 ± 45	677 ± 5	879 ± 94
Pool 3	908 ± 52	795 ± 25	637 ± 28	892 ± 111
Pool 4	446 ± 57	978 ± 71	855 ± 53	855 ± 11
Pool 5	461 ± 39	1,004± 29	784 ± 41	885 ± 29
Pool 6	1059± 46	843 ± 54	637 ± 31	739 ± 11
Pool 7	525 ± 11	1,458 ± 70	1,877 ± 32	1,974 ± 52
Rec. OspA	1,115 ± 82	650 ± 79	325 ± 26	348 ± 13

5×10^5 splenocytes were incubated with respective antigen/mitogen or without stimulation (cells alone) in triplicate for 102 hrs and then pulsed with one μCi of ^{3}H-thymidine per well. Data is presented as mean CPM ± S.E obtained after harvesting the cells 18 hrs later and counting in liquid scintillation counter.

Splenocytes from mice infested with pathogen free ticks or injected with PBS served as matched controls for tick transmitted and needle inoculated infection, respectively.

Table C. *In vitro* proliferative responses of splenocytes from BALB/c mice infected with *B. burgdorferi*, either by tick transmission or by needle inoculation, to Concanavalin A (Con A), Lipopolysaccharide (LPS), whole spirochete extract from type strain B31 (WSE B31), whole spirochete extract from OspA and B negative mutant (WSE AB def.), fraction pools from preparative SDS-PAGE of WSE (AB def., Pool 1 through 7), and recombinant OspA (Rec. OspA). Two weeks post infection, Three day assay

Treatment	Tick trans. inf.	Needle inoc. inf.	Uninfected ticks	PBS inject.
Con A	17,686 ± 1102	21,583± 466	21,000 ± 637	13,638 ± 410
LPS	3,109 ± 68	2,460 ± 147	3,722 ± 81	2,333 ± 31
Cells Alone	684± 36	304 ± 10	332 ± 14	249 ± 7
WSE (B31)	5,321± 95	3,708 ± 233	3,463 ± 173	2,425 ± 87
WSE (AB def.)	5,166 ± 95	4,416 ± 438	4,208 ± 252	2,165 ± 254
Pool 1	1,720 ± 39	1,032 ± 52	956 ± 20	802 ± 164
Pool 2	437 ± 19	351 ± 17	303 ± 14	280 ± 5
Pool 3	1,352 ± 110	1,163 ± 203	1,303 ± 88	636 ± 30
Pool 4	934 ± 60	529 ± 47	303 ± 18	353 ± 57
Pool 5	759 ± 41	341± 26	350 ± 51	274 ± 25
Pool 6	465± 15	306 ± 8	321 ± 12	216 ± 8
Pool 7	5,409 ± 225	4,160 ± 246	5,009 ± 218	3,078 ± 17
Rec. OspA	930± 12	829 ± 116	729 ± 56	460 ± 30

5×10^5 splenocytes were incubated with respective antigen/mitogen or without stimulation (cells alone) in triplicate for 54 hrs and then pulsed with one μCi of ^{3}H-thymidine per well. Data is presented as mean CPM ± S.E obtained after harvesting the cells 18 hrs later and counting in liquid scintillation counter.

Splenocytes from mice infested with pathogen free ticks or injected with PBS served as matched controls for tick transmitted and needle inoculated infection, respectively.

Table D. *In vitro* proliferative responses of splenocytes from BALB/c mice infected with *B. burgdorferi*, either by tick transmission or by needle inoculation, to Concanavalin A (Con A), Lipopolysaccharide (LPS), whole spirochete extract from type strain B31 (WSE B31), whole spirochete extract from OspA and B negative mutant (WSE AB def.), fraction pools from preparative SDS-PAGE of WSE (AB def., Pool 1 through 7), and recombinant OspA (Rec. OspA). Two weeks post infection, Five day assay

Treatment	Tick trans. inf.	Needle inoc. inf.	Uninfected ticks	PBS inject.
Con A	14,671± 1870	14,642 ± 1300	22,769 ± 1034	20,977 ± 1079
LPS	1,983 ± 88	1,445 ± 110	3,212 ± 161	2,633 ± 188
Cells Alone	638 ± 36	404 ± 22	483 ± 12	294 ± 15
WSE (B31)	2,024± 147	1,516 ± 120	1,485 ± 86	883 ± 74
WSE (AB def.)	2,204 ± 86	1,973 ± 106	1,552 ± 188	996 ± 87
Pool 1	1,349 ± 167	1,326 ± 116	1,007 ± 21	720 ± 41
Pool 2	1,009 ± 155	308 ± 32	391 ± 33	149 ± 9
Pool 3	1,031 ± 129	1,429 ± 55	1,045 ± 47	714 ± 80
Pool 4	2,226± 149	391 ± 30	316 ± 29	190 ± 28
Pool 5	953 ± 74	382± 57	344 ± 29	267 ± 16
Pool 6	435± 25	255 ± 37	358 ± 21	146 ± 6
Pool 7	2,501 ± 102	2,336 ± 143	2,567 ± 256	2,715± 365
Rec. OspA	352 ± 48	531 ± 59	236 ± 17	163 ± 7

5×10^5 splenocytes were incubated with respective antigen/mitogen or without stimulation (cells alone) in triplicate for 102 hrs and then pulsed with one μCi of ^{3}H-thymidine per well. Data is presented as mean CPM ± S.E obtained after harvesting the cells 18 hrs later and counting in liquid scintillation counter.

Splenocytes from mice infested with pathogen free ticks or injected with PBS served as matched controls for tick transmitted and needle inoculated infection, respectively.

Table E. *In vitro* proliferative responses of splenocytes from BALB/c mice infected with *B. burgdorferi*, either by tick transmission or by needle inoculation, to Concanavalin A (Con A), Lipopolysaccharide (LPS), whole spirochete extract from type strain B31 (WSE B31), whole spirochete extract from OspA and B negative mutant (WSE AB def.), fraction pools from preparative SDS-PAGE of WSE (AB def., Pool 1 through 7), and recombinant OspA (Rec. OspA). Four weeks post infection, three and five day assay

	Three day assay		Five day assay	
Treatment	**Tick trans. inf.**	**Needle inoc. inf.**	**Tick trans. inf.**	**Needle inoc. inf.**
Con A	5,689± 141	14,043 ± 186	5,468 ± 362	18,012 ± 144
LPS	1,044 ± 65	1,415 ± 48	482± 12	1,226 ± 36
Cells Alone	183 ± 11	252 ± 9	168 ± 9	300 ± 13
WSE (B31)	1,244± 30	2,840 ± 81	339 ± 27	1,229 ± 103
WSE (AB def.)	1,097 ± 96	2,656 ± 154	347 ± 47	1,534± 116
Pool 1	454 ± 52	1,029 ± 60	351 ± 36	801 ± 63
Pool 2	224 ± 14	447 ± 49	219 ± 18	561 ± 60
Pool 3	553 ± 14	1,097 ± 80	247 ± 3	925 ± 100
Pool 4	278± 16	612 ± 49	242 ± 42	1,023 ± 73
Pool 5	235 ± 12	380 ± 13	190 ± 7	388 ± 33
Pool 6	217 ± 26	507 ± 43	273 ± 16	548 ± 41
Pool 7	2,065 ± 132	4,926 ± 167	1,290 ± 53	3,173 ± 197
Rec. OspA	602 ± 200	649 ± 5	216 ± 27	326 ± 41

5×10^5 splenocytes were incubated with respective antigen/mitogen or without stimulation (cells alone) in triplicate for 54 and 102 hrs and then pulsed with one μCi of ^{3}H-thymidine per well. Data is presented as mean CPM ± S.E obtained after harvesting the cells 18 hrs later and counting in liquid scintillation counter.
Splenocytes from mice infested with pathogen free ticks or injected with PBS which served as matched controls for tick transmitted and needle inoculated infection, respectively, were not employed.

Table F. *In vitro* proliferative responses of splenocytes from BALB/c mice infected with *B. burgdorferi*, either by tick transmission or by needle inoculation, to Concanavalin A (Con A), Lipopolysaccharide (LPS), whole spirochete extract from type strain B31 (WSE B31), whole spirochete extract from OspA and B negative mutant (WSE AB def.), fraction pool from preparative SDS-PAGE of WSE (AB def., Pool 7), and recombinant OspA (Rec. OspA). Eight weeks post infection, Three day assay

Treatment	Tick trans. inf.	Needle inoc. inf.	Uninfected ticks	PBS inject.
Con A	97,757 ± 1,621	72,935 ± 1,826	92,318 ± 402	77,982 ± 3,064
LPS	36,208 ± 766	38,721 ± 674	38,823 ± 980	38,063 ± 1,166
Cells Alone	1,152 ± 21	516 ± 38	1,057 ± 112	832 ± 17
WSE (B31)	28,902 ± 1195	34,160 ± 1,363	30,757 ± 1,460	26,244 ± 287
WSE (AB def.)	17,627 ± 346	15,702 ± 1,171	20,440 ± 1,149	12,890 ± 8
Pool 7	28,289 ± 249	23,240 ± 654	22,122 ± 593	20,598 ± 551
Rec. OspA	17,635 ± 61	17,692 ± 1,447	11,516 ± 769	9,517 ± 240

5×10^5 splenocytes were incubated with respective antigen/mitogen or without stimulation (cells alone) in triplicate for 54 hrs and then pulsed with one μCi of ^{3}H-thymidine per well. Data is presented as mean CPM ± S.E obtained after harvesting the cells 18 hrs later and counting in liquid scintillation counter.

Splenocytes from mice infested with pathogen free ticks or injected with PBS served as matched controls for tick transmitted and needle inoculated infection, respectively. Fraction pools one through six were not tested.

Table G. *In vitro* proliferative responses of splenocytes from BALB/c mice infected with *B. burgdorferi*, either by tick transmission or by needle inoculation, to Concanavalin A (Con A), Lipopolysaccharide (LPS), whole spirochete extract from type strain B31 (WSE B31), whole spirochete extract from OspA and B negative mutant (WSE AB def.), fraction pools from preparative SDS-PAGE of WSE (AB def., Pool 1 through 7), and recombinant OspA (Rec. OspA). Eight weeks post infection, five day assay

Treatment	Tick trans. inf.	Needle inoc. inf.	Uninfected ticks	PBS inject.
Con A	180,448 ± 5,746	88,088 ± 6,900	142,951 ± 6,666	113,061 ± 4,548
LPS	16,394 ± 1,177	17,138 ± 303	15,673 ± 179	20,732 ± 1,076
Cells Alone	3,865 ± 160	1,746 ± 104	2,598 ± 229	2,074± 211
WSE (B31)	11,224 ± 323	24,096 ± 1,451	11,212 ± 279	6,618 ± 561
WSE (AB def.)	7,582 ± 181	8,539 ± 713	6,657 ± 51	3,520 ± 162
Pool 7	10,806 ± 884	9,279 ± 663	7,200 ± 195	3,857 ± 140
Rec. OspA	4,948 ± 117	3,763 ± 241	1,341 ± 92	1,239 ± 33

5×10^5 splenocytes were incubated with respective antigen/mitogen or without stimulation (cells alone) in triplicate for 102 hrs and then pulsed with one μCi of ^{3}H-thymidine per well. Data is presented as mean CPM ± S.E obtained after harvesting the cells 18 hrs later and counting in liquid scintillation counter.

Splenocytes from mice infested with pathogen free ticks or injected with PBS served as matched controls for tick transmitted and needle inoculated infection, respectively. Fraction pools one through six were not tested.

Table H. *In vitro* proliferative responses of splenocytes from BALB/c mice infected with *B. burgdorferi*, either by tick transmission or by needle inoculation, to Concanavalin A (Con A), Lipopolysaccharide (LPS), whole spirochete extract from type strain B31 (WSE B31), whole spirochete extract from OspA and B negative mutant (WSE AB def.), fraction pools from preparative SDS-PAGE of WSE (AB def., Pool 1 through 7), and recombinant OspA (Rec. OspA). 12 weeks post infection, Three day assay

Treatment	Tick trans. inf.	Needle inoc. inf.	Uninfected ticks	PBS inject.
Con A	10,494 ± 141	13,467± 597	26,745 ± 801	12,076 ± 658
LPS	1,175 ± 48	1,328 ± 18	1,760 ± 38	1,042 ± 46
Cells Alone	236 ± 12	220 ± 8	274 ± 36	181 ± 7
WSE (B31)	2,505 ± 137	3,576 ± 65	3,833 ± 309	2,912 ± 253
WSE (AB def.)	1,727 ± 47	3,091 ± 173	1,739 ± 0.4	1,570 ± 171
Pool 1	244 ± 15	213 ± 13	339 ± 30	251 ± 11
Pool 2	213 ± 13	235 ± 39	268 ± 11	230 ± 24
Pool 3	265 ± 12	340 ± 16	381 ± 20	316 ± 16
Pool 4	152 ± 9	210 ± 12	233 ± 20	181 ± 5
Pool 5	193 ± 8	205± 8	294 ± 54	238 ± 32
Pool 6	145± 11	225 ± 33	232 ± 10	1,144 ± 7
Pool 7	801 ± 28	1,434 ± 29	1,806 ± 144	219 ± 12
Rec. OspA	611 ± 43	1,049 ± 35	1,147 ± 26	847 ± 39

5×10^5 splenocytes were incubated with respective antigen/mitogen or without stimulation (cells alone) in triplicate for 54 hrs and then pulsed with one μCi of ^{3}H-thymidine per well. Data is presented as mean CPM ± S.E obtained after harvesting the cells 18 hrs later and counting in liquid scintillation counter.

Splenocytes from mice infested with pathogen free ticks or injected with PBS served as matched controls for tick transmitted and needle inoculated infection, respectively.

Table I. *In vitro* proliferative responses of splenocytes from BALB/c mice infected with *B. burgdorferi*, either by tick transmission or by needle inoculation, to Concanavalin A (Con A), Lipopolysaccharide (LPS), whole spirochete extract from type strain B31 (WSE B31), whole spirochete extract from OspA and B negative mutant (WSE AB def.), fraction pools from preparative SDS-PAGE of WSE (AB def., Pool 1 through 7), and recombinant OspA (Rec. OspA). 12 weeks post infection, Five day assay

Treatment	Tick trans. inf.	Needle inoc. inf.	Uninfected ticks	PBS inject.
Con A	12,995 ± 384	15,866± 985	18,105 ± 926	20,016 ± 1,133
LPS	1,494 ± 84	1,704 ± 163	1,903 ± 68	1,180 ± 117
Cells Alone	195 ± 19	204 ± 13	211 ± 11	186 ± 9
WSE (B31)	1,515 ± 219	2,402 ± 167	3,272 ± 99	1,530 ± 187
WSE (AB def.)	1,184 ± 8	1,838 ± 177	2,310 ± 169	1,609 ± 88
Pool 1	127 ± 5	164 ± 11	296 ± 15	174 ± 23
Pool 2	115 ± 17	206 ± 39	167 ± 20	202 ± 25
Pool 3	148 ± 15	238 ± 27	361 ± 56	182 ± 21
Pool 4	153 ± 28	173 ± 29	219 ± 34	147 ± 11
Pool 5	124 ± 5	132 ± 12	162 ± 8	117 ± 8
Pool 6	143 ± 19	133 ± 13	174 ± 14	248 ± 102
Pool 7	603 ± 47	1,046 ± 66	758 ± 33	830 ± 57
Rec. OspA	355 ± 13	579 ± 64	1,030 ± 101	620 ± 13

5×10^5 splenocytes were incubated with respective antigen/mitogen or without stimulation (cells alone) in triplicate for 102 hrs and then pulsed with one μCi of ^{3}H-thymidine per well. Data is presented as mean CPM ± S.E obtained after harvesting the cells 18 hrs later and counting in liquid scintillation counter.

Splenocytes from mice infested with pathogen free ticks or injected with PBS served as matched controls for tick transmitted and needle inoculated infection, respectively.

VITA

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Thesis: COMPARATIVE STUDIES ON IMMUNE RESPONSES TO TICK TRANSMITTED AND NEEDLE INOCULATED INFECTION OF *BORRELIA BURGDORFERI* IN BALB/C MICE

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