Journal of Spirochetal and Tick-borne Diseases

March 1996 Volume 3 • Number 1

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Etiologic Agent

Borrelia burgdorferi are helically shaped bacteria with dimensions of 0.18 to 0.25 microns by 4 to 30 microns. Seven or eleven periplasmic flagella are located at each end of the cell. Lyme disease is an infection caused by *B. burgdorferi* which is transmitted in the bite of *Ixodes* and *Amblyomma* ticks.

The Disease and Diagnosis

People who acquire Lyme disease do so after an infected tick attaches itself to the skin of its host for about 24 hours. As the tick starts to take in a meal of blood, *B. burgdorferi* spirochetes in the mid gut begin to multiply. They then cross into the tick's circulation, migrate to the salivary glands and pass with saliva into the host. Your patients can establish a strong line of defense just by checking for ticks every day. Any ticks that are discovered can then be analyzed for *B. burgdorferi* by using the Lyme Disease Alert System™ for rapid results. Ticks received by the laboratory have their mid gut tissues removed and cultured in New Jersey Laboratories' specially modified Barbour-Stonner-Kelly medium. Cultures are examined later for spirochetes using phase contrast microscopy. All positive isolates are then immunostained with four monoclonal antibodies for *B. burgdorferi*, outer surface protein A (OSPA), OSPB (34kD), flagellin (41kD), and P39 (39kD). Antisera to *B. burgdorferi* is then reacted with the isolate and observed via immunoflourescent assay techniques.

Human Granulocytic Ehrlichiosis (HGE)

New Jersey Laboratories has also developed a method for the detection of HGE bacteria in ticks. Again, the tick mid guts are removed and cultured using a canine macrophage line. The *Ehrlichieae* are non-motile, gram-negative cocci found in the membrane -lined cytoplasmic vacuoles; usually they form inclusion bodies (morulae). After culturing, the cells are stained and examined for morulae. Positive cultures are confirmed using IFA techniques.

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GUEST EDITORIAL

MAJOR ARTICLES

DEPARTMENTS

The Journal of the Lyme Disease Foundation

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Editorial content: Topics relating to understanding disease mechanisms and the application of better diagnostic techniques and treatment strategies for all individuals suffering from spirochetal and tick-borne diseases. ***Letters* to the Editor** in the form of correspondence related to material published in the Journal or some aspects of spirochetal and tick-borne diseases may be submitted. Such letters, if related to work previously published in the journal, will be referred to the author of the original work for a response.

Guest Editorial

A Compendium on Animal Models of Lyme Disease

Mario T. Philipp, PhD

This issue of the *Journal of Spirochetal and Tick-borne Diseases* focuses on how research using animal models has contributed to our current understanding of Lyme borreliosis. Attempts to identify suitable animal models began soon after the spirochetal etiology of the disease was discovered. The rabbit was among the very first species investigated,[1-4] in part because of its susceptibility to infection with *Treponema pallidum*, the agent of syphilis. At the time, emphasis was placed on finding signs of arthritis. None were observed and the model was not pursued further despite the occurrence of erythema migrans (EM) in a small number of animals.[3,4] *Mutatis mutandis*, it is now possible to reproducibly induce skin lesions in the rabbit which have the appearance and the histopathologic features of human EM. These observations, as well as experiments on infection-derived protective immunity, which the rabbit is able to elicit,[5] are reviewed herein by Jim Miller and his colleagues. Because "skin is home for *Borrelia burgdorferi*," to quote Stephen Barthold, or a preferred site for spirochetal localization, it is important to have available an inexpensive model with which to elucidate the basis for the persistence of infection in this organ. Several factors that could contribute to determine whether an infection will or will not elicit EM, such as tick species, *B burgdorferi* (*sensu lato*) isolate/species, or infectious dose, also could be studied easily in the rabbit. The diagnostic importance of EM and the relatively high proportion of Lyme disease cases that are not foretold by this clinical sign are a context in which such studies should be very significant.

Hamsters were initially described as susceptible to a fully disseminated *B burgdorferi* infection in the absence of disease signs.[6] They were subsequently used to investigate antimicrobials in vivo and to assess the role of antibody in protective immunity by means of serum-transfer experiments.[7] Thus were established what are still basic tenets of protective immunity to *B burgdorferi*, ie, that antibody is its key mediator and that antibody is effective if present at the time of spirochetal transmission but in most cases not when administered thereafter.[7] When it became apparent that the development and/or type of arthritis in hamsters depended—surely among other factors as well—on whether spirochetes were confronted by an undeveloped[8] or impaired[9] immune system, the site of inoculation (hind paws[8]), and on whether the animals had been immunologically sensitized with killed spirochetes prior to inoculation,[10] the model became useful to investigate factors leading to severe destructive arthritis. This aspect is reviewed by Ron Schell and his colleagues, who emphasize that the propensity of the hamster to develop this form of intense arthritis may be especially useful to assess adverse effects of vaccines.

Whenever possible, an attempt should be made to enlist the mouse as a model for human disease. The availability of a multitude of inbred strains with well-defined genetic backgrounds, including strains with immunological defects attained either by mutation or genetic manipulation, and a veritable plethora of recombinant cytokines and their antibodies, as well as antibodies directed against functional cell-surface antigens and phenotypic markers, is unparalleled by any other animal model. Within the last 5 years, researchers in the field of Lyme borreliosis have tapped these riches to explore genetic determinants of susceptibility to infection and disease and the role of the different components of the immune system in curtailing or blocking infection, or causing exacerbation, attenuation, or resolution of disease. Steven Barthold and Marcus Simon with his colleagues thoroughly and critically review these aspects. The laboratory mouse, as well as mouse species that serve as natural reservoirs for *B burgdorferi*, also have been employed to investigate features of the tick-host interface that may be relevant to spirochetal transmission and dissemination, such as the modulation of the host immune response by tick saliva.

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This and other issues pertaining to transmission of *B burgdorferi sensu lato* by reservoir hosts are reviewed by Klaus Kurtenbach.

All models are inherently imperfect and thus their effective utilization must rely on the skillful framing of a significant and answerable experimental question. The discovery that invasion of the brain of *scid* mice by *Borrelia turicatae* correlates with the expression of a variable membrane protein (Vmp) of 23 kDa is an example of a potentially very important finding (small Vmps are homologous to the OspC lipoproteins of *B burgdorferi*) that was gleaned from the *B turicatae-scid* mouse model. The model is reviewed by Alan Barbour.

When assessing the safety of a therapeutic or prophylactic protocol for a human disease, it is germane to utilize an animal model that is both taxonomically as close as possible to the human, and able to reproduce the most morbid signs of the disease at issue, in this case Lyme arthritis and neuroborreliosis. The rhesus monkey satisfies these requirements[11-14] and a paper on the safety and the immunogenicity of OspA vaccines in this animal model is included. Finally, the important question of where does *B burgdorferi* lurk after dissemination is addressed by Max Appel and his colleagues, using the dog as a model.

I would like to thank all of the contributors for their timely efforts, and especially Martina Ziska for generating the opportunity to bring this volume together. I hope it will be useful.

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The Rabbit as a Model for the Study of Lyme Disease Pathogenesis and Immunity—A Review

James N. Miller, PhD; Denise M. Foley, PhD; Jon T. Skare, PhD; Cheryl I. Champion, PhD; Ellen S. Shang, BA; David R. Blanco, PhD; and Michael A. Lovett, MD, PhD

ABSTRACT

Culture-positive erythema migrans (EM) lesions have been induced consistently and reproducibly on the clipped backs of adult, New Zealand white rabbits following the intradermal injection of *Borrelia burgdorferi*. Studies utilizing the B31 strain have shown that EM can be produced after as many as 47 in vitro passages, although the degree of induction is less consistent than with lower passaged organisms. Infiltrating lymphocytes, plasma cells, and macrophages with perivascular cuffing, characteristic of the human EM lesion, were observed within the superficial and deep layers of the EM lesion. Skin infection and dissemination to the popliteal lymph nodes, joint tissue, and spinal cord were also produced and persisted for 8 to 10 weeks after infection. Ultimate clearance of EM and local and disseminated infection less than 3 months after intradermal infection with the development of complete immunity to reinfection are striking features that distinguish the rabbit model from both the monkey and other small animal models. In studies utilizing the B31 strain, the extent of protective immunity was directly related to the extent of prior in vitro passage; passage 4 organisms induced complete protection, while passage 27 and 47 organisms induced corresponding lesser degrees of acquired resistance. Serum from a rabbit completely immune to challenge with the low passage B31 *B burgdorferi* and adsorbed with an avirulent ATCC B31 strain isolate, was used to identify outer membrane (OM)-spanning protein antigens specific for the virulent strain. On the basis of 2-D gel electrophoresis and immunoblot analysis of TX-114 detergent phase OM proteins, seven antigens, including OspD, were found only in passage 10 organisms, two were common to both passage 10 and passage 48 organisms, and one was found only in passage 48 organisms; the acronym, Oms^{vsa}, was used to designate OM-spanning proteins that are only virulent-strain associated.

Immunoelectron microscopy studies using the adsorbed serum paralleled the immunoblot analysis and provided compelling evidence for the presence of antibodies directed against virulent strain associated antigens with putative virulence and protective immunogenic properties. Ongoing and future studies in the rabbit relevant to the understanding of Lyme disease pathogenesis and immunology include: the elucidation of pathogenetic and immune mechanisms that mediate the course of the disease process, clearance of the infection, and the establishment and maintenance of both endogenous and exogenous acquired resistance; evaluation of vaccinogens; and comparison of the rabbit response to needle vs infected tick inoculation.

Key words: Lyme disease, rabbit model, pathogenesis, immunity

INTRODUCTION

Lyme disease, the most common vector-borne disease in the United States,[1] is characterized by the appearance of distinctive erythema migrans (EM) skin lesions in 60% to 85% of patients.[2] Early and late clinical manifestations reflecting dissemination to visceral targets include arthritis, neurological manifestations, lymphadenopathy, and carditis.[2-8] Implicit in the development of control measures for this disease is a complete understanding of the cellular and molecular mechanisms of pathogenesis and immunology operative during the course of the disease. Of imminent importance is the elucidation of those mechanisms at play during and immediately following initial contact between the spirochete and host leading

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to the development of the EM lesion(s), dissemination, and latency vs reactivation as opposed to complete and lasting immunity. Animal models in which these parameters can be simulated provides the opportunity to explore essential pathogenetic and immune mechanisms responsible for the entire spectrum of infection and disease as well as those factors that influence the development of acquired resistance.

RODENT AND RHESUS MONKEY MODELS: ADVANTAGES AND LIMITATIONS

In contrast to the rhesus monkey,[9] the small and practical rodents used in experimental investigations lack the ability to produce EM lesions.[10-18] This, together with the chronic persistence of disseminated infection in each of these animal models,[10-19] precludes their use in attempting to elucidate the mechanisms underlying the development of infection-derived acquired resistance against both EM and dissemination.

The characteristic perivascular lymphocytic, plasma cell, and macrophage infiltration observed in human EM lesions and infected viscera[20] has been observed in the EM lesions of the rhesus monkey[9] and in the target organs of dissemination in both the monkey and rodent models.[13-15,18,19,21] However, the role of immune cells during the course of EM development and healing, and during persistence and disappearance of spirochetes from the skin, has not been addressed. Indeed, the continued persistence of spirochetes in rodent and monkey visceral tissues precludes the conduct of studies in these animals designed to determine the immune mechanisms that mediate healing, clearance, and the development of acquired resistance following infection.

Rodents have provided seemingly satisfactory models for evaluating the efficacy of Osp components, *Borrelia burgdorferi* bacterin, and a *B burgdorferi* mutant lacking OspA and OspB as vaccines.[22-29] Passive protection and correlative complement-dependent borreliacidal assays appear to provide compelling evidence for the major role of humoral immune mechanisms operative in protective immunity against disseminated infection in rodents following vaccination or infection.[23,30,31] Unfortunately, the use of "tests of challenge" not capable of evaluating protection against the development of EM lesions places a severe restriction on the ability to interpret accurately both the efficacy of the potential vaccinogen and operative cellular *and* humoral immune mechanisms responsible for acquired resistance.

PREVIOUS ATTEMPTS TO DEVELOP A RABBIT MODEL

Prior to the studies conducted in our laboratory, attempts to induce EM reproducibly in the rabbit were unsuccessful. Although erythematous lesions had been observed, *B burgdorferi* could not be cultured from putative EM, nor could the characteristic histopathology of EM be demonstrated. Burgdorfer and his colleagues described the appearance of annular, erythematous lesions in New Zealand white rabbits after being fed upon by *Ixodes scapularis* infected with *B burgdorferi*.[32] These lesions, however, rarely appeared at the site of tick feeding and did not develop until 4 to 12 weeks after tick engorgement.

Kornblatt and his coworkers attempted to produce EM on the clipped backs of New Zealand white rabbits using three methods: by feeding presumptively infected *I scapularis*, by injecting infected tick organ homogenates, or by injecting a culture of *B burgdorferi* strain G39/40, passage 50.[33] Of the 33 rabbits inoculated by these various methods, one of four rabbits fed upon by infected ticks developed an erythematous skin lesion at the site of tick attachment that persisted for 3 days; however, silver stains of the lesion biopsies showed spirochetes but cultures were negative. Of the 10 rabbits injected intradermally with tick organ homogenates, one developed an erythematous, indurated lesion at two of the three inoculated sites; these lesions, which appeared in 2 days and persisted for 6 days, were silver stain and culture negative. None of the 19 rabbits inoculated both intradermally and intravenously with either tick organ homogenates or the G39/40 strain developed EM.

DISTINCTIVE FEATURES RELATING TO PATHOGENESIS AND IMMUNOLOGY IN THE RABBIT

In recently published studies, we have described the consistent and reproducible induction of culture-positive EM lesions on the clipped backs of adult, New Zealand white rabbits following the intradermal injection of six different low-passaged virulent strains of *B burgdorferi* with concentrations ranging from 10^3 to 10^7 organisms per site[34] (Tables 1 and 2, Fig 1). Studies using the B31 strain have shown that EM can be produced after as many as 47 in vitro passages, although the degree of lesion induction is less consistent than with lower passaged organisms (Table 1). EM lesions at the injection site appeared within 5 days after infection and persisted an average of 7 days. Infiltrating lymphocytes, plasma cells, and macrophages with perivascular cuffing, characteristic of the human EM lesion, were observed within the superficial and deep dermal layers of the EM lesion.[34] Skin infection and dissemination to the popliteal lymph nodes, joint tissue, and spinal cord also were produced[34] (Tables 3-A, 3-B, and 4).

Further studies demonstrated that one strain of *B burgdorferi* could be cultured from the skin, joint tissue,

Table 1
EM After ID Inoculation of Rabbits With **B burgdorferi** *B31 Strain*

	Inoculum*				
	10^7	10^6	10^5	10^4	10^3
	EM/Total sites				
In vitro passage					
4	8/8	8/8	8/8	ND	ND
8	8/8	8/8	8/8	8/8	8/8
27	8/8	8/8	8/8	8/8	8/8
47	8/8	8/8	4/8	8/8	6/8
ATCC B31					
Avirulent control	0/8	ND	ND	ND	ND

**Two rabbits were injected at four sites for each inoculum tested.*
ND, not done.
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Table 2
Production of EM After ID Inoculation of Rabbits With Seven Strains of **B burgdorferi**

		Lesions	
Strain*	**EM/sites**	**Time of appearance**	**Duration**
		d[‡]	*d*[§]
B31	24/24	3-8	1-9
N40	24/24	4-5	5-19
SH-2-82	24/24	4-7	5-15
HB19	24/24	4-7	2-7
297	24/24	4-5	1-6
Ca-2-87	24/24	4-6	5-9
ECM-NY-86	11/24	5	5-7
Heat-killed	0/8	N/A	N/A
ATCC Avirulent B31	0/8	N/A	N/A

**Four rabbits were inoculated per strain with 10^7 organisms per site. Controls include two animals inoculated with 2 x 10^7 heat-killed organisms (56°C for 45 min) from each strain and two animals that were inoculated with 4 x 10^7 ATCC B31. Bladder isolates from infected mice were used at in vitro passage 1. ‡Mean = 5±1; §mean = 7±3. N/A, not applicable.*
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and spinal cord; one strain from skin and joint tissue; two strains from skin and spinal cord; and two from the skin only (Table 4). Although preliminary, these findings are in accord with the possibility that certain strains of *B burgdorferi* may have a specific affinity for the skin, central nervous sysem, and/or joints. Organisms persisted in the skin and viscera for 8 to 10 weeks after infection[34] (Table 3-B, Fig 2, unpublished data).

This ultimate clearance of EM as well as local and disseminated infection within 3 months of intradermal infection is a striking feature that distinguishes the rabbit model from both the monkey and other small animal models. Resolution of the infection provides a convenient means for studying the ontogeny of those immune mechanisms that may contribute to EM development and those responsible for clearing the infection. In preliminary studies, we have shown that within 12 hours of the intradermal inoculation of normal and immune rabbits with 10^7 B31 passage 4 *B burgdorferi*, an infiltration of polymorphonuclear leucocytes appears at the site of inoculation in both the naive and immune animals and persists for up to 4 days.[35]

In the immune animals in which EM lesions failed to develop during a 28-day observation period, no further

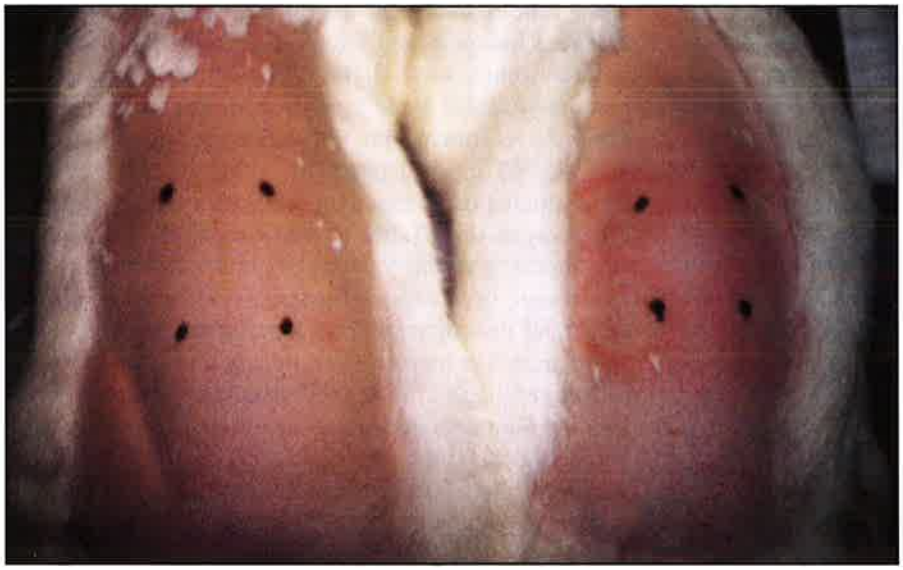

Fig 1. Erythema migrans in the New Zealand white rabbit. Right—typical EM target lesions at each of 4 sites 9 days after ID injection with 10^7 B burgdorferi, *B31 strain passage 4. Left—note the absence of lesions following ID injection of 10^7 avirulent* B burgdorferi, *B31 strain (ATCC).*
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Table 3
Dissemination of B burgdorferi *B31 Passage 4 After ID Inoculation*

(A) Intradermal inoculation of a total of 4 x 10^7 *B burgdorferi* B31

Tissues	3 & 5 h	24 h	1 wk	2 wk	3 wk	4 wk
Skin	1/2	2/2	2/2	2/2	2/2	2/2
Ear	0/2	0/2	1/2	1/2	1/2	1/2
Blood	0/2	0/2	0/2	0/2	0/2	0/2
Spleen	0/2	2/2	0/2	0/2	0/2	0/2
Liver	0/2	2/2	0/2	0/2	0/2	0/2
Heart	0/2	1/2	0/2	0/2	0/2	0/2
Popliteal nodes	0/4	0/4	1/2	1/2	2/2	1/2
Stifle joint tissue	ND	ND	ND	1/1	1/2	1/2
Synovial fluid	ND	ND	ND	1/2	0/2	0/2
Conjunctiva	ND	ND	ND	2/2	0/2	0/2
Spinal cord	0/4	0/4	2/2	2/2	2/2	2/2

(B) Intradermal inoculation of a total of 6 x 10^3 *B burgdorferi* B31

Tissues	1 wk	2 wk	3 wk	4 wk	5 wk	8 wk
Skin	9/10	3/3	6/7	3/3	3/4	1/4
Popliteal nodes	ND	2/3	ND	1/3	ND	0/4
Stifle joint tissue	ND	3/3	ND	2/3	ND	0/4
Spinal cord	ND	1/3	ND	0/3	ND	0/4

Data is expressed as No. of rabbits/total No. of rabbits. ND, not done.
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Table 4
Dissemination of Six Strains of B burgdorferi *4 Weeks After ID Inoculation*

	Strain					
	N40	SH-2-82	HB 19	297	CA-2-87	ECM-NY-86
Tissues						
Skin	2/2	2/2	2/2	2/2	2/2	2/2
Popliteal nodes	2/2	1/2	0/2	1/2	0/2	0/2
Stifle joint tissue	1/2	0/2	2/2	0/2	0/2	0/2
Synovial fluid	0/2	0/2	0/2	0/2	0/2	0/2
Spinal cord	2/2	1/2	0/2	1/2	0/2	0/2

For all strains two rabbits were inoculated at each of eight sites with 10^7 organisms per site. Strains used were obtained from skin biopsy cultures of previously infected rabbits and used at in vitro passage 4. Data are expressed as positive rabbits/total rabbits.
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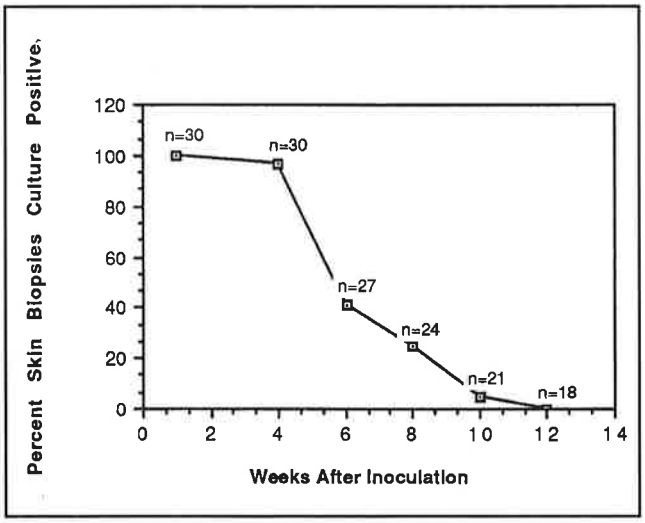

Fig 2. Clearance of B Burgdorferi *B31 from the skin after intradermal injection. Skin biopsies were obtained at each timepoint and cultured in BSK II medium. Graph marker, percentage of culture positive rabbits. Reprinted with permission of Rockefeller Press.*

infiltration was observed and skin biopsies at the site of inoculation were culture negative. In contrast, a lymphocytic, plasma cell, and macrophage infiltration corresponding to the appearance of culture-positive EM lesions occurred in the naive animals and persisted beyond the time of EM healing and skin resolution. These data suggest that the polymorphonuclear leucocytic host response to *B burgdorferi* is not capable of destroying the organism, that the chronic inflammatory

Table 5
Homologous Challenge of Rabbits Infected ~5 Months Previously With **B burgdorferi** *B31*

Initial injection		Challenge dose*			
		10^7	10^6	10^5	Culture results
B burgdorferi	**Inoculum**	**EM/total sites**			**at day 5-7‡**
Noninfectious	N/A	6/6	6/6	6/6	+
Heat-killed	4×10^7	3/4	4/4	4/4	+
Lab strain	4×10^7	7/8	6/8	4/8	+
Passage 4	4×10^7	0/4	0/4	0/4	–
	4×10^6	0/2	0/2	0/2	–
	4×10^5	0/2	0/2	0/2	–
	4×10^4	0/2	0/2	0/2	–
	4×10^3	0/4	0/4	0/4	–
Passage 8	4×10^7	0/2	0/2	0/2	–
	4×10^6	2/4	0/2	0/2	–
	4×10^5	1/2	0/2	0/2	–
	4×10^4	0/4	0/4	0/4	–
	4×10^3	3/4	2/4	3/4	–
Passage 27	4×10^7	2/4	2/4	2/4	–
	4×10^6	2/2	0/2	0/2	–
	4×10^5	2/4	2/4	1/4	–
	4×10^4	0/2	0/2	0/2	–
	4×10^3	2/4	2/4	2/4	–
Passage 47	4×10^7	2/2	0/2	0/2	+
	4×10^4	0/2	0/2	0/2	+
	4×10^3	2/2	2/2	2/2	+

**Rabbits were challenged with passage 4 B31. EM lesions were developed at 5–7 d after challenge. ‡Negative biopsied skin cultures obtained at 5–7 d were repeated and found negative at 18, 32, 46, 67, and 81 d after challenge. N/A, not applicable.*
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response may contribute to both EM formation as well as subsequent healing of the lesion and clearance of the spirochetes, and that complete infection-derived immunity is predominately or exclusively antibody mediated.

Another striking feature of the rabbit model is the development of immunity to reinfection in animals challenged with homologous strains of *B burgdorferi*. Within 5 months of initial intradermal infection with three strains of 4×10^7 low-passaged *B burgdorferi*, at a time when both the skin and viscera are cleared of spirochetes, subsequent challenge with as many as 8×10^7 passage 4 homologous spirochetes resulted in no evidence of dermal or disseminated infection[34] (Tables 5 and 6). In the case of the B31 strain, the extent of the protective immunity induced by untreated infection is directly related to the extent of prior in vitro passage[34] (Table 5). Only passage 4 could induce complete protection against EM and dermal infection with as few as 4×10^3 spirochetes.

In contrast, initial infection with passage 27 organisms led to partial protection against EM and complete protection against skin infection. Passage 47 organisms induced no protection against skin infection and partial protection against EM. These data imply that certain determinants required for the production of acquired resistance are lost during in vitro passage. Further, these differences between low and high passage strain B31 may allow the identification of proteins responsible for virulence and/or the induction of complete immunity by the low-passaged spirochetes. Indeed, Skare and his coworkers already have reported significant changes in outer membrane vesicle TX-114 hydrophobic phase proteins during sequential in vitro passage.[36]

Inasmuch as our recent focus has been on structure-

Table 6
***Homologous ID Challenge of Rabbits Infected 5 Months Previously with 4 x 10^7 Low Passaged Strains of* B burgdorferi**

Strain	EM/ Total sites	Skin biopsy cultures: positive rabbits	Visceral cultures: positive rabbits
positive rabbits			
B31	0/16	0/2	0/2
N40	0/16	0/2	0/2
SH-2-82	0/16	0/2	0/2
HB19	8/16	0/2	0/2
297	8/16	0/2	0/2
CA-2-87	0/16	2/2	0/2
ECM-NY-86	0/16	0/2	0/2
Controls			
Heat killed ATCC	16/16	2/2	1/2
Avirulent B31	16/16	212	1/2
Noninfected	99/112*	14/14	8/14‡

Two rabbits were challenged at each of eight sites with 10^7 organisms/site. Strains used for challenge were obtained from skin biopsy cultures of previously infected rabbits and used at in vitro passage 4. Data shown for punch biopsy cultures were obtained at 7–11 d after challenge.
*Similar data were obtained at days 21 and 28. Controls include the low passage B31 challenge of those animals previously inoculated with either 2 x 10^7 heat-killed organisms from each strain or given 4 x 10^7 avirulent ATCC B31, and inoculation of two noninfected animals for each strain. Visceral cultures examined in each rabbit were spinal cord, popliteal lymph nodes, and stifle joint tissue. *13 of 16 sites inoculated with the ECM-NY-86 strain failed to develop lesions. ‡See Table 4. Reprinted with permission of Rockefeller Press.*

Table 7
Hydrophobic OMV Antigens Recognized by Immunoblotting with Adsorbed Sera (Molecular Mass in kD)

Passage 10 B31	Passage 48 B31	Avirulent ATCC B31	Description
16.5		16.5	Oms
18b	18b	18b	Oms, C
19.5a*	19.5a*	19.5a*	Oms, C
19.5c		19.5c	Oms
25c		25c	Oms
28	28		Oms, vsa‡
29a (Osp D)			L, vsa§
29b	29b		Oms, vsa‡
	30		Oms, vsa‡‡
35b			Oms, vsa§
39		39	EF**
40a			Oms, vsa§
40b			Oms, vsa§
40c†			Oms, vsa§
42†			Oms, vsa§
70†			Oms, vsa§

*L, lipoprotein; Oms, candidate outer membrane-spanning protein; C, protein common to all OMV preparations; *for proteins with identical molecular masses, a designates the most acidic protein and the subsequent letters indicate proteins that are more basic; ‡vsa, virulent strain associated protein present in passage 10 and 48 OMV preparations (Figs 3A and B); §vsa, virulent strain associated protein present only in passage 10 OMV preparation (Fig 3A); ‡‡vsa, virulent strain associated protein present only in passage 48 OMV preparation (Fig 3B); †not observed in passage 10 OMV 2-D gold stain; **endoflagellin (EF). Reprinted with permission of Rockefeller Press.*

function relationships of outer membrane (OM)-spanning proteins, the isolation and purification of *B burgdorferi* OM vesicles (OMV) with porin activity by Skare and his coworkers[36] provided the opportunity to conduct further studies designed to identify, isolate, and purify potentially significant protein antigens from OMV. Serum from a rabbit completely immune to challenge with low-passage B31 *B burgdorferi* was adsorbed with an avirulent ATCC B31 strain isolate to remove antibodies that recognize antigens common to both the virulent and avirulent spirochete.[34] The resulting adsorbed serum contained antibodies specifically enriched for low-passage, virulent *B burgdorferi* antigens and was significantly depleted of antibodies found in the avirulent ATCC B31 strain.

Separation of Triton X-114 detergent phase OM proteins from B31 passage 10 (found to be equivalent to passage 4 in terms of EM induction), B31 passage 48, and the avirulent B31 strain by 2-D gel electrophoresis and subsequent probing of immunoblots with the adsorbed serum, revealed seven antigens found only in passage 10, one of which was identified as OspD36 (Table 7, Fig 3). Two antigens were common to both the passage 10 and passage 48 B31; one was found in passage 48.

The authors have chosen the acronym, Oms, to designate the OM-spanning proteins shared by virulent and avirulent B31 *B burgdorferi*, and Oms^{vsa} for Oms that are virulent-strain associated only. Immunoelectron microscopy using the adsorbed serum paralleled the immunoblot analyses of each of these three isolates[36] (Table 8). The adsorbed serum was found to be more reactive with the virulent isolates than the avirulent isolate.

The observation that the adsorbed serum contains

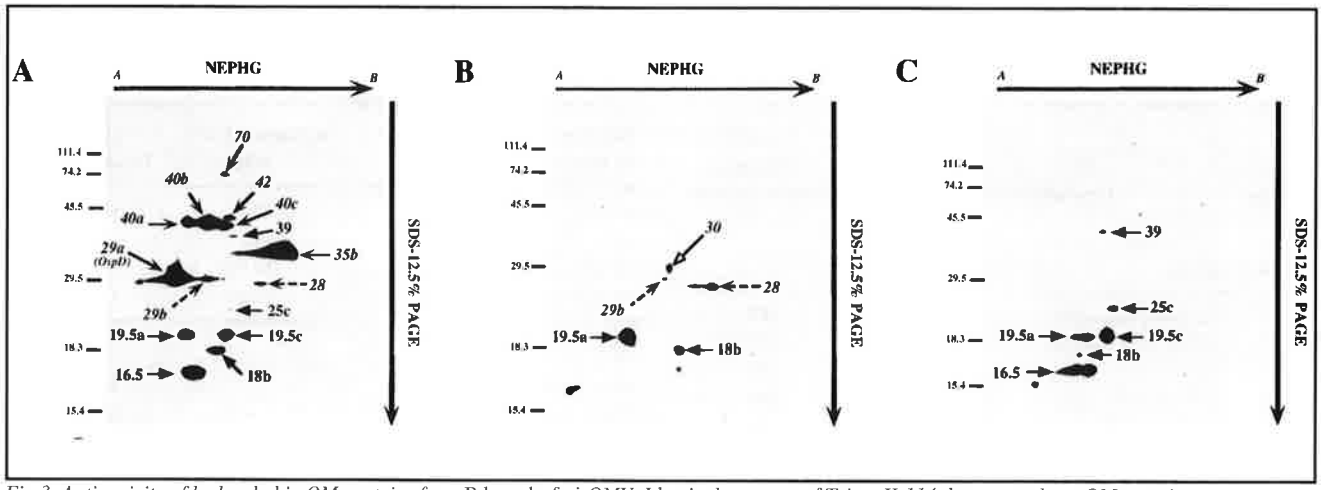

Fig 3. Antigenicity of hydrophobic OM proteins from B burgdorferi *OMV. Identical amounts of Triton X-114 detergent phase OM proteins were separated by 2-D gel electrophoresis and immunoblotted. A and B indicate the acidic and basic ends of the nonequilibrium pH gel (NEPHG), respectively. The immunoblot was incubated in infection-derived rabbit serum adsorbed with avirulent ATCC B31* B burgdorferi. *Lower case letters after the numbers listed distinguish proteins of identical molecular mass with different pI values. The acidic-most spot is designated a; subsequent letter assignments refer to spots with more basic pI values. Numbers to the left indicate the molecular masses of protein markers (kD). (A) OMV derived from $5x10^9$ B31 passage 10* B burgdorferi. *(B) OMV derived from $5x10^9$ B31 passage 48 B. burgdorferi. (C) OMV derived from $5x10^9$ B31 avirulent ATCC* B burgdorferi. *Reprinted with permission of Rockefeller Press.*

antibodies that specifically and preferentially bind the surface of the virulent B31 passage 1 *B burgdorferi* relative to the B31 passage 50 and avirulent ATCC B31 spirochetes provides compelling evidence for presence of antibodies in the adsorbed serum directed specifically against virulent strain-associated antigens with putative virulence and protective immunogenic properties. Of equal significance is the fact that, consistent with the decrease in proteins observed with the antigenic profiles of the OMV preparations from the virulent and avirulent B31 passages[36] (Table 7, Fig 3), the number of gold particles observed for *B burgdorferi* whole cells decreased with increasing in vitro passage[36] (Table 8).

Taken together, the data accrued from the studies using adsorbed serum indicate that Oms^{vsa} are candidate proteins relevant to the pathogenesis and immunology of experimental Lyme disease. Although the Oms^{vsa} described in the studies of Skare and his coworkers are proteins found in *B burgdorferi* after in vitro cultivation, it is conceivable that protein molecules are expressed only in vivo in environments unique to the vertebrate and invertebrate hosts of *B burgdorferi.*[36] Consistent with this possibility is the evidence that a supercoiled plasmid-encoded protein designated EppA and the lipoprotein OspF are expressed only in vivo.[37,38]

ONGOING AND FUTURE STUDIES

The ability to induce EM, skin and disseminated infection, and infection-derived immunity in the rabbit has provided the opportunity to add new dimensions to our approach to the study of Lyme-disease pathogenesis and immunology. The mechanisms that mediate the initial events leading to the production of EM, dissemination, latency, and immunity remain poorly understood. Apparently, the influx of polymorphonuclear leucocytes immediately following infection cannot eradicate the organisms and thus prevent their establishment in the host. The unrestricted multiplication of spirochetes leads to the induction of EM lesions and skin and visceral infections as a result of the contribution of *B burgdorferi* virulence factors and/or the infiltrating chronic inflammatory response to the organism.

Studies along these lines are ongoing or have been designed to clone, sequence, and express the genes that code for the Oms^{vsa} proteins; to examine their putative role as virulence determinants; and to correlate the appearance, persistence, and disappearance of EM and infection with the presence vs absence of T and B lymphocytes, plasma cells, and macrophages.

If T lymphocytes are detected, the presence of $CD8^+$ and $CD4^+$, characterized as TH1 and TH2, will be determined. Experiments to determine the relative distribution of peripheral B and T lymphocytes as well as levels of humoral antibody in relationship to the presence, location, and clearance of the spirochetes also will be conducted.

Although clearance of the initial infection may be mediated by both humoral and cellular mechanisms, the absence of any evidence to indicate that an inflammatory response occurs following the challenge of completely

Table 8
Recognition of Surface Exposed Proteins by IEM Using Preimmune, Unadsorbed Immune, and Adsorbed Immune Sera

Strain*	Basal (Preimmune)	Unadsorbed‡ (Immune)	Adsorbed‡ (Immune)
	gold particles per per μm length§		
Passage 1	4.24±2.69 (n = 11)	167.8±67.511 (n = 7)	29.0±11.4† (n = 8)
Passage 50	1.04±0.47 (n = 5)	90.0±17.9‡‡ (n = 5)	6.41±4.23† (n = 10)
Avirulent ATCC	0.39±0.18 (n = 5)	85.3±35.3‡‡ (n = 5)	1.58±0.60† (n = 10)

**Whole mount IEM was conducted with* B burgdorferi *B31 passage 1, passage 50, and ATCC avirulent whole cells. ‡Unadsorbed immune serum was obtained from a rabbit infected with passage 4 B31* B burgdorferi.[34] *Adsorbed serum was obtained by incubating the unadsorbed serum described above with ATCC avirulent B31* B burgdorferi. *§Values represent the average number of gold particles observed per mm length of the* B burgdorferi *B31 analyzed ±SD; n refers to the number of fields of each sample analyzed under the electron microscope. ‡‡Wilcoxon ranked sum test indicated a significant difference (P = 0.039) when the unadsorbed immune serum was reacted with three strains tested. †Wilcoxon ranked sum test indicated a significant difference (P = 0.0001) when the adsorbed immune serum was reacted with three strains tested.*
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immune animals strongly implies that exogenous resistance is mediated exclusively by humoral antibody. Passive protection, opsonophagocytic, complement-dependent borreliacidal, and adherence and invasin inhibition assays have been planned in an effort to confirm this hypothesis.

A highly significant advantage of the rabbit vs the rodent models is the fact that the former permits the definition of vaccine efficacy against EM as well as skin and disseminated infection. If a test vaccinogen exhibits a high degree of—but not complete—protection against skin and disseminated infection, an evaluation must be made of its ability to protect against EM in the susceptible animals. Protection against only EM will result in an undetected, "masked" latent state with the potential for reactivation of the disease. Studies to determine the protective immunogenicity of purified Oms^{vsa} proteins as well as a recombinant OspA vaccine being evaluated in humans are underway.

The rabbit model using needle inoculation has provided and continues to provide data that contribute significantly to our understanding of the pathogenesis and immunology of Lyme disease. However, the necessity to compare and contrast the events following intrdermal infection with the natural method of tick transmission, mandates our planned approach to the elucidation of mechanisms of host-infected tick interaction.

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Hamster Model of Lyme Borreliosis

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ABSTRACT

The hamster is an excellent model to study the immune responses to infection or vaccination with *Borrelia burgdorferi sensu lato*. When immunocompetent adult hamsters are infected with *B burgdorferi sensu lato* isolates 297 or C-1-11, they develop arthritis. Severe destructive arthritis can also be elicited in vaccinated hamsters after challenge with isolates from the six seroprotective groups of *B burgdorferi sensu lato*. The development of severe destructive arthritis is dependent on CD4+ T lymphocytes. The hamster's propensity to develop arthritis, especially severe destructive arthritis, is an effective way to evaluate vaccines for adverse effects. In addition, the hamster mimicked the vaccine response of humans to a recombinant subunit vaccine.

Key words: Lyme borreliosis, hamster, arthritis, *Borrelia burgdorferi*

INTRODUCTION

Animal models of Lyme borreliosis are extremely important for elucidating the mechanisms of pathogenesis and immunity. These models increase our knowledge and understanding of the basic infectious process and assist in developing strategies to prevent infection and disease in humans. Unfortunately, no single animal model mimics all the pathological and clinical manifestations associated with Lyme borreliosis. Skin lesions that resemble human erythema migrans develop in monkeys, rabbits, and guinea pigs.[1-4] Moderate or chronic progressive arthritis (synovitis) develops in mice and young dogs,[5-7] while severe chronic arthritis can be induced in severe combined immunodeficiency (*scid*) mice and irradiated hamsters[8,9] after infection with *Borrelia burgdorferi sensu lato*. Carditis, nephritis, and hepatitis also can be detected in some of these animal models infected with the Lyme spirochete.[1,10] However, detection of neuroborreliosis with these animal models has not been reported. In this issue of the Journal, neuroborreliosis is reported as a feature of the canine model.

The compartmentalization of clinical features along with the diversity of animal models is beneficial for studying Lyme borreliosis. Each animal model enhances and strengthens the ability of investigators to define common pathological and immunological principles that are likely to occur in humans. Divergent findings are even more important and generally have a far greater impact on defining mechanisms of resistance or other pathological mechanisms induced by infection or vaccination. It is imperative then to define the uniqueness of each animal model to obtain a broad and comprehensive picture of pathological events that might occur in humans.

When adult inbred LSH hamsters were injected in the hind paws with 10^6 *B burgdorferi* sensu lato, clinical manifestations of Lyme arthritis were induced (Fig 1). Inflammation or swelling of the hind paws could be detected 7 days after infection, peaked on day 10, and gradually decreased. At week 1, the tibiotarsal, intertarsal, and interphalangeal joints showed evidence of acute inflammation. The synovial lining was hypertrophic and hyperplastic, and areas of ulceration were easily

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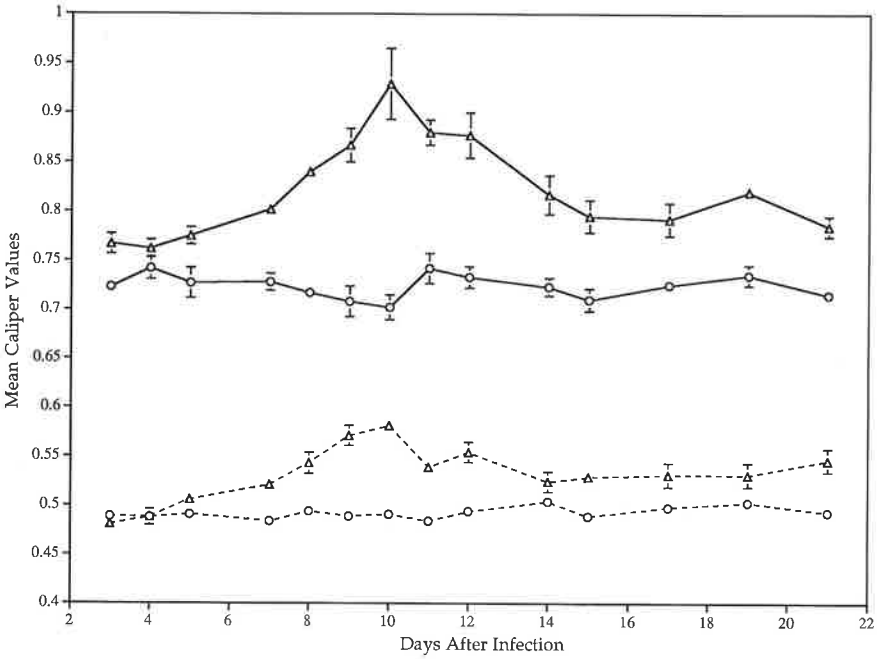

Fig 1. Swelling of the hind paws of adult hamsters (————) and 6-week-old C3H/HeJ mice (- - - - -) with (Δ) or without (O) infection with Borrelia burgdorferi sensu stricto *isolate 297 contained in Barbour-Stoenner-Kelly medium. Controls (m) were inoculated with Barbour-Stoenner-Kelly medium.*

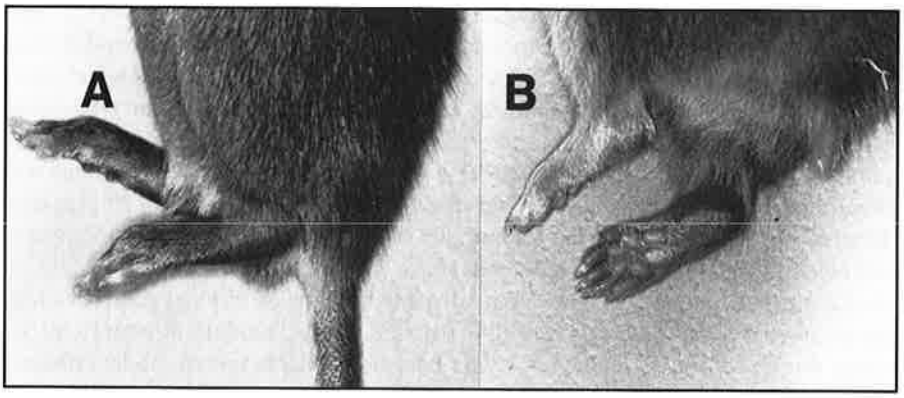

Fig 2. Appearance of the hind paws of a 6-week-old C3H/HeJ mouse (A) and an adult immunocompetent hamster (B) after infection with B burgdorferi sensu stricto *isolate 297. Photograph was taken 10 days after infection at the peak interval of swelling (see Fig 1). The photograph of the mouse was magnified three times.*

detected. Adherent fibrin protruded into the joint spaces and was associated with inflammatory cells, especially neutrophils. The neutrophils also penetrated the subsynovial connective tissue and periarticular structures including ligaments, tendons, tendon sheaths, fibrous capsule, and periosteum. Spirochetes (>20 in high power field) were readily observed in the subsynovial tissues. By week 3 after infection, the number of spirochetes had greatly decreased and the inflammatory response was resolving. A chronic synovitis characterized by hypertrophic villi, focal erosions of articular cartilage and subsynovial mononuclear infiltrate persisted for approximately 1 year.

Similar responses were obtained in mice. When 6-week-old C3H/HeJ mice were infected subcutaneously in the hind paws with 10^6 *B burgdorferi sensu stricto* isolate 297, minor swelling of the hind paws was detected (Fig 1). Swelling occurred in approximately 7 days after infection, peaked on day 10, and gradually waned. In addition, no significant difference in swelling of the hind paws was detected between hamsters and mice when the percentage of swelling was determined. This measurement eliminated the differences in size of the paws for determination of the amount of swelling. When mice were infected with other isolates of *B burgdorferi* sensu lato, the same degree of swelling of the hind paws was detected. Use of older C3H/HeJ mice, however, abrogated the ability of *B burgdorferi* to induce swelling of the hind paws. These results demonstrate that immunocompetent adult hamsters and young C3H/HeJ mice make a similar clinical response (synovitis) to infection with *B burgdorferi* (Fig 2A and B). Furthermore, only minor differences are detected in Western immunoblot profiles when sera from infected hamsters and mice are compared.

A major difference, however, is the ability of hamsters

to develop adverse effects, especially severe destructive arthritis, when vaccinated with a whole-cell preparation of Formalin-inactivated *B burgdorferi sensu lato*. All seroprotective groups[11] or genospecies[12] of *B burgdorferi sensu lato* can prime or elicit the severe destructive arthritis. The elicitation of arthritis is prevented only when the vaccinates are challenged with the homologous (vaccine-specific) isolate. However, severe destructive arthritis can still be elicited in the vaccinates when the homologous challenge is given before protective borreliacidal antibody has developed. Hamsters can be primed (vaccinated) for the development of severe destructive arthritis with different preparations of whole *B burgdorferi sensu lato* including those exposed to heat, treated with antimicrobial agents, or prepared by freezing and thawing. In addition, priming of hamsters with these preparations is not dependent upon the type of adjuvant. One of the adjuvants (alum) we used is approved for human and veterinary usage.

Specifically, five groups of three hamsters each were vaccinated with Formalin-inactivated *B burgdorferi sensu stricto* isolate C-1-11 in aluminum hydroxide gel.[13] At 1, 3, 5, 7, and 9 weeks after vaccination, hamsters were challenged in the hind paws with *B burgdorferi sensu stricto* isolate C-1-11. Hamsters vaccinated for 5 weeks or less developed severe destructive arthritis (Fig 3). The peak swelling was approximately two-fold greater (plethysmograph value of 1.2) than the peak swelling (plethysmograph value of 0.6) detected in normal immunocompetent hamsters challenged with *B burgdorferi sensu stricto* isolate C-1-11. Control noninfected hamsters had a plethysmograph value of approximately 0.4. By contrast, hamsters challenged homologous spirochete after the fifth week of vaccination failed to develop severe destructive arthritis. Nonvaccinated hamsters or hamsters vaccinated with Barbour-Stoenner-Kelly medium also failed to develop any swelling of the hind paws. In other experiments, severe destructive arthritis was elicited in vaccinated hamsters challenged intradermally, intramuscularly and intraperitoneally. The onset of arthritis, however, was delayed by approximately 20 days.

When the above experiments were repeated using Formalin-inactivated spirochetes from the six seroprotective groups of *B burgdorferi sensu lato*, similar results were obtained. Lovrich et al[11] identified six seroprotective groups among a large number of *B burgdorferi sensu lato* isolates, including *B burgdorferi sensu stricto*, *Borrelia garinii*, and *Borrelia afzelii*. All the isolates from the seroprotective groups could prime (vaccinate) or elicit by infection or challenge the severe destructive arthritis. If priming and elicitation were performed with the homologous spirochete, development of severe destructive arthritis was prevented. Presently, we are determining whether

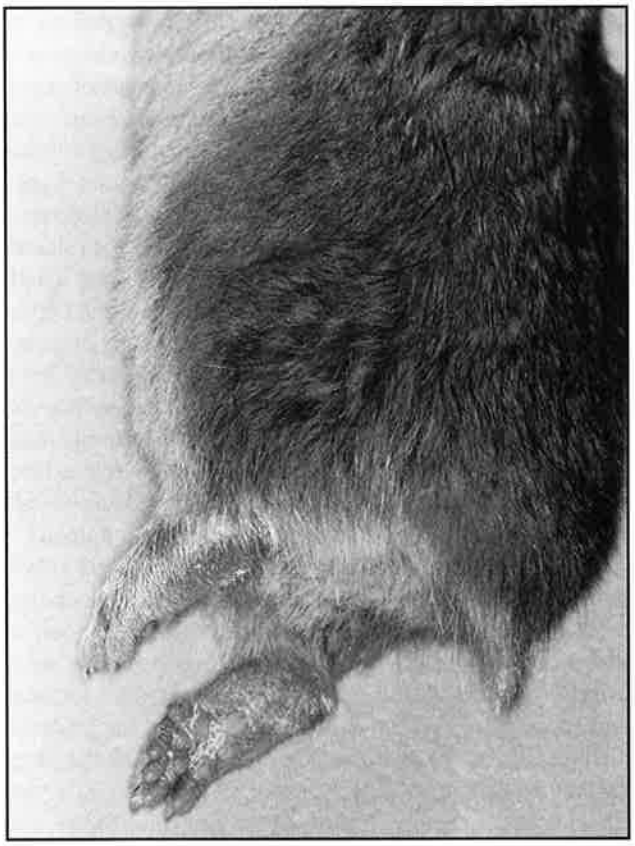

Fig 3. Appearance of the hind paws of a hamster vaccinated with Formalin-inactivated B burgdorferi sensu stricto *isolate C-1-11 in aluminum hydroxide gel and challenged with the same viable isolate. Photograph was taken twelve days after infection. The plethysmograph value of swelling for the hind paws of vaccinated and challenged hamsters was three times the plethysmograph value of the control group. Severe destructive arthritis can be elicited with all isolates of* B burgdorferi sensu lato *including isolate C-1-11.*

homologous challenge will elicit severe destructive arthritis once vaccine-specific borreliacidal antibody has decreased. Most disconcerting is that severe destructive arthritis still develops when vaccinates are challenged with other isolates of the genomic groups of *B burgdorferi sensu lato* despite high levels of vaccine specific borreliacidal antibody. This suggests that Lyme borreliosis vaccines must be composed of several isolates or their subunits to induce a comprehensive borreliacidal antibody response to prevent the development of arthritis.

This is the first documentation of development of adverse effects after vaccination against infection with *B burgdorferi sensu lato*. There are several explanations why severe destructive arthritis was not reported previously. Although vaccination of dogs[14,15] and other experimental animals[16-18] has occurred, vaccinates are commonly challenged with the homologous *B burgdorferi sensu lato* isolate. Generally, challenge occurs at the time of peak

borreliacidal antibody activity and not during periods of antibody development or decline. Another explanation is that vaccinates are not routinely challenged with isolates of *B burgdorferi sensu lato* belonging to different serogroups.[11] We elicited severe destructive arthritis in vaccinates challenged with nonhomologous Lyme spirochetes in the presence or absence of homologous borreliacidal antibody.[13] A third explanation is the route of challenge. When other routes of challenge are used, besides subcutaneous infection of the hind paws, the onset of severe destructive arthritis is delayed 20 days or more. The late development of arthritis may not have been observed by investigators. A fourth explanation may be that the hamster is uniquely susceptible to priming with the arthritigenic proteins of *B burgdorferi* sensu lato. Even if other animal models of Lyme borreliosis do not develop the severe destructive arthritis, the hamster's propensity to develop this adverse effect may lead to better recognition of the arthritigenic agents and their mechanism for priming and elicitation of arthritis. Gondolf et al[19] has shown that the outer surface proteins of *B burgdorferi* are potent arthritogens in rats. The hamster and rat may be ideal models to test vaccines for potential adverse effects. In addition, the hamster, unlike the other animal models, may closely resemble a subset of Lyme patients that develop chronic Lyme arthritis. Steere and colleagues[20] have shown that 10% of Lyme patients develop continuous joint inflammation for 1 year or longer. This is consistent with the course of severe destructive arthritis in the hamster.

We also showed that the adverse effects were not mediated by humoral factors.[13] Severe destructive arthritis was evoked in the presence or absence of high titers of borreliacidal protective antibody and total anti-*B burgdorferi* antibody. Even repeated passive transfers of serum from hamsters vaccinated with Formalin-inactivated *B burgdorferi sensu stricto* isolate C-1-11 did not induce arthritis in the presence or absence of challenge with the homologous isolate or nonhomologous isolates. Fikrig et al[21] also showed that vaccination with OspA did not enhance arthritis but caused its resolution.

We suggested that cell-mediated immunity was responsible for the development and elicitation of severe destructive arthritis.[13] Other studies, however, have stressed that T lymphocytes play only a minor role in the pathogenesis of Lyme borreliosis.[22,23] Recently, we presented unequivocal evidence that *B burgdorferi*-specific T lymphocytes are responsible for the induction of severe destructive arthritis.[24,25] Hamsters were vaccinated with Formalin-inactivated *B burgdorferi sensu stricto* C-1-11 in adjuvant. When naive recipient hamsters were infused with T lymphocytes from the vaccinated hamsters, they developed severe destructive arthritis after challenge with *B burgdorferi sensu stricto* isolate C-1-11. Swelling of the hind paws was detected 6 days after challenge and increased rapidly with peak swelling occurring 14 to 16 days after challenge. By contrast, recipients infused with normal T lymphocytes and challenged with isolate C-1-11 developed only slight swelling of the hind paws. The swelling developed slowly (day 10) and resolved quickly compared to the swelling detected in hamsters infused with immune T lymphocytes. In addition, the swelling detected in recipients of normal T lymphocytes and challenged with *B burgdorferi sensu stricto* isolate C-1-11 was similar to the swelling detected in normal hamsters infected with isolate C-1-11.

As further support that T lymphocytes play an important role in the development of severe destructive arthritis, we treated vaccinated hamsters with a rat monoclonal antibody, GK 1.5, that recognizes the CD4 molecule on hamster helper T lymphocytes.[25] When vaccinated hamsters were depleted of CD4+ T lymphocytes by administration of the monoclonal antibody and challenged with isolate C-1-11, they failed to develop severe destructive arthritis. Similarly, nonvaccinated hamsters with or without depletion of CD4+ T lymphocytes failed to develop severe destructive arthritis. However, severe destructive arthritis was readily evoked in hamsters vaccinated with the whole cell vaccine preparation and challenged with *B burgdorferi sensu stricto* isolates 297 or C-1-11.

A direct role for CD4+ T lymphocytes in the development of severe destructive arthritis is not surprising. CD4+ T lymphocytes have been shown to participate in the development of rheumatoid arthritis[26-29] in humans and induction of collagen arthritis in mice.[30] What is surprising is the extent that CD4+ T lymphocytes are involved in the development of severe destructive arthritis. Treatment of vaccinated hamsters with monoclonal antibody GK 1.5 completely prevented the development of arthritis. When administration of the monoclonal antibody was discontinued, severe destructive arthritis was easily detected. The mechanism by which *B burgdorferi* specific CD4+ T lymphocytes are involved in the pathogenesis of arthritis is unknown. Additional experiments are needed to define the contribution of the subsets of CD4+ T lymphocytes, cytokines, and antigen presenting cells.

Keane-Myers and Nickell[31,32] also have shown a role for T lymphocytes in the development of arthritis (synovitis) in mice. They showed that depletion of subsets of T lymphocytes altered the pathogenesis of *B burgdorferi* infection in C3H/HeJ and BALB/c mice. Depletion of CD4+ T lymphocytes enhanced the severity of arthritis (synovitis) suggesting that CD8+ T lymphocytes may be responsible for the exacerbation of arthritis. Although these results conflict with ours, they demonstrate the importance of T lymphocytes in the development of Lyme

arthritis. An explanation may be that CD4+ T lymphocytes play a greater role in the induction of severe destructive arthritis than they do in controlling the mild arthritis that is detected in young mice and adult hamsters after infection with *B burgdorferi sensu lato*. When nonvaccinated hamsters are depleted of CD4+ T lymphocytes and infected with *B burgdorferi*, they develop only synovitis. This suggests that CD8+ T lymphocytes are responsible for the development of synovitis in nonvaccinated hamsters. Collectively, these studies do illustrate the importance of cell mediated immunity, specifically T lymphocytes, in controlling or preventing the induction of events leading to the development of synovitis in nonvaccinated hamsters and severe destructive arthritis in vaccinated hamsters.

Our results suggest that another approach besides whole spirochetes is needed for the development of a Lyme borreliosis vaccine. Several *B burgdorferi* sensu lato proteins, including OspA,[11,21,33] OspB,[33,34] OspC,[17,33] and the 39 kDa protein,[35] have been shown to kill *B burgdorferi* in vitro or induce protection in animals against challenge with the Lyme disease spirochete. Of these proteins, OspA has emerged as the leading vaccine candidate. Presently, OspA is being tested for safety and immunogenicity in human field trials.[36,37]

We evaluated the ability of a recombinant OspA (rOsp) vaccine (Connaught Laboratories Inc, Swiftwater, Penn), to induce and maintain sustained levels of borreliacidal antibody. Johnson et al[38] and Schmitz et al[39,40] established the important role of antibodies that kill *B burgdorferi* for providing protection against infection. Therefore, we monitored the ability of humans and hamsters to produce borreliacidal antibodies after vaccination with rOspA.[41] Briefly, female and male volunteers were vaccinated with a placebo, 1 μg, 5 μg, 10 μg, or 30 μg of rOspA. A booster vaccination was administered 30 days after the primary vaccination. Borreliacidal antibodies were not detected consistently in individuals vaccinated with 30 μg of rOspA or less. Borreliacidal activity was detected in 0%, 40%, 80%, and 81% of vaccinates 60 days after vaccination with 1, 5, 10, or 30 μg of rOspA, respectively. Even at the peak borreliacidal antibody response (60 days) the titers (range 0 to 80) of cidal antibodies were low and varied greatly. Only one individual had detectable borreliacidal activity 120 days after vaccination.

When hamsters were vaccinated with 120 μg of the rOspA vaccine and boosted 28 days after the primary vaccination, a similar borreliacidal antibody response was detected. Peak cidal activity was detected 6 weeks after vaccination (2 weeks after booster) and waned rapidly. No borreliacidal activity was detected 30 weeks after vaccination. When serum was obtained from vaccinated humans and hamsters at peak borreliacidal activity and incubated with different seroprotective group isolates,[11] only the homologous (vaccine) isolate (B31) was killed. These results were confirmed in vivo by challenging rOspA vaccinated hamsters with four different seroprotective group isolates. Again, vaccinated hamsters were protected only against challenge from the homologous vaccine isolate.

These results are discouraging and unimpressive. This suggests that the rOspA is a poor immunogen, despite lipidation, for both humans and hamsters. Although borreliacidal antibody was induced, the titers were low and cidal activity waned rapidly. In addition, the rOspA induced a borreliacidal antibody response that was restricted to the homologous challenge. Vaccinates would not be protected from infection with other isolates of the seroprotective groups of *B burgdorferi sensu lato.*

These results confirmed the findings of Lovrich et al.[11] They showed that antisera generated against the rOspA of *B burgdorferi sensu stricto* isolates S-1-10 and C-1-11 (seroprotective groups 1 and 2, respectively), *B burgdorferi* BV1 (seroprotective group 4), and *B garinii* LV4 (seroprotective group 5) could kill the homologous spirochete but not other spirochetes. Antisera against the rOspA of *B afzelii* PKo (seroprotective group 6) and *B burgdorferi* sensu lato LV5 (seroprotective group 3) failed to kill the homologous isolate. These results demonstrate that rOspA has considerable immunologic and molecular heterogeneity and that a single OspA preparation will not induce comprehensive protection against all isolates of *B burgdorferi sensu lato.*

To these difficulties must be added the ability of *B burgdorferi* to alter its outer surface proteins during attachment and feeding. Schwan et al and colleagues[42,43] showed that *B burgdorferi* down-regulates the expression of OspA and begins rapid synthesis of OspC. Even though anti-OspA serum has been shown to sterilize ticks infected with the Lyme spirochete,[44,45] the period of attachment and level of circulating anti-OspA may be extremely critical for borreliacidal activity to occur. If *B burgdorferi* rapidly up-regulates OspC while down-regulating OspA, some spirochetes still may survive and be available for transmission to the host. Collectively, these studies suggest that combinations of *B burgdorferi sensu lato* proteins will be ultimately required for a comprehensive vaccine. The composition of the vaccine, however, is confounded by the immunologic and molecular heterogeneity of the major outer surface proteins that can induce protection against the Lyme spirochete.

Another concern is the reinventing of *B burgdorferi* to develop a comprehensive vaccine. As more and more protective antigens are added to the composition of the vaccine, the ability of these combinations of proteins and

other antigens to induce or elicit adverse effects may increase. Likewise, the immunogenicity of these antigens must be proven. An adjuvant may be required to induce and maintain sustained levels of borreliacidal antibodies. The vaccine containing adjuvant may also enhance the potential for adverse effects, like severe destructive arthritis. Another approach for vaccination would be to use whole spirochetes that induce broad cross-protection and eliminate those antigens that are responsible for the induction of arthritis or other possible autoreactive pathological responses.[46-48] Although this approach is not popular, it may be more feasible because of the immunogenic and molecular heterogeneity of the vaccine protein candidates and the number of antigens that can induce borreliacidal antibodies.

In conclusion, the hamster is an excellent model to study the immune responses to infection with *B burgdorferi*. Subsets of T lymphocytes can be distinguished and their interaction with other immune response cells can be determined. In addition, the humoral response of hamsters mimics the antibody response of humans to infection or vaccination. More importantly, the hamster's propensity to develop adverse effects, specifically severe destructive arthritis, is an effective way to define the antigen(s) responsible for arthritis and evaluating vaccines for adverse effects. Each animal model presents unique opportunities, and they should be explored to obtain a comprehensive picture of the types of responses humans may develop.

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Lyme Borreliosis in the Laboratory Mouse

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ABSTRACT

Humans are only one among many species susceptible to *Borrelia burgdorferi* infection and disease. The catholic tastes of *B burgdorferi* for a variety of mammalian hosts is a fortunate circumstance for scientific investigation, as it has allowed experimental infection of a number of laboratory animal species, including mice,[1-4] rats,[5,6] hamsters,[7,8] gerbils,[9] Peromyscus mice,[10,11] guinea pigs,[12,13] rabbits,[14] dogs,[15] and monkeys.[16,17] All of these model systems have proven useful, but the mouse is the most utilitarian, as mice are relatively inexpensive to purchase and maintain, are microbiologically and genetically defined, and are embellished with immunogenetic tools that allow maximal manipulation of the model. Like humans, mice develop multisystemic infection, manifested as musculoskeletal and cardiovascular disease. Disease resolves and recurs episodically over the course of persistent infection. Unlike humans, mice do not develop erythema migrans or neuroborreliosis, and there is no correlate of the chronic unremitting disease that occurs in a minority of Lyme disease patients. Admittedly, Lyme borreliosis in the mouse is not totally parallel with all features of human Lyme disease, but there are important common elements amenable to experimental analysis. This review will provide an overview of work with the mouse model, as well as discuss the relationship of findings in the mouse with human Lyme disease.

Key words: Lyme borreliosis, laboratory mice

REVIEW

Infection and dose response

Mice can be infected by syringe with cultured spirochetes, by tick-borne infection, or by transplantation of tissues from other infected mice. All three methods have proven useful for experimental studies, and the important point is that the immune response appears to be identical by all methods of infection, as long as the initial infecting dose is small enough to be immunologically subliminal until subsequent generations of spirochetes replicate and disseminate in the host. The ensuing immune response does not discriminate among these different forms of spirochetes, but spirochetes in culture, ticks, and tissues are remarkably different antigenically, and their vulnerability to host immunity depends upon their adaptive state. This must be taken into account in experimental design.

Mice can be efficiently infected with very low doses of spirochetes if given intradermally, and dissemination and disease occur regardless of dose. The intradermal syringe-borne median infectious dose (ID_{50}) was identical (10 spirochetes) for genetically disease-susceptible C3H mice and disease-resistant BALB mice. In fact, the ID_{50} for intradermal inoculation was shown to be 10 to 100-fold lower than intraperitoneal inoculation.[2,18] Infectious dose was not influenced by incubation temperature of spirochetes, and the serologic response to infection with spirochetes grown at different temperatures was identical.[18] Dose has proven to be a critical factor in assessing immune response to *B burgdorferi*. When mice were inoculated intradermally with 10^7, 10^6, 10^4, 10^2, or 10^1 heat-killed or viable spirochetes, significantly different immunoblot responses were found, depending upon dose and viability of the inoculum (Fig 1). Mice inoculated with high doses of spirochetes (>10^6) developed antibody to outer surface protein (Osp) A and B, whereas mice inoculated with lower doses did not,[18,19] which

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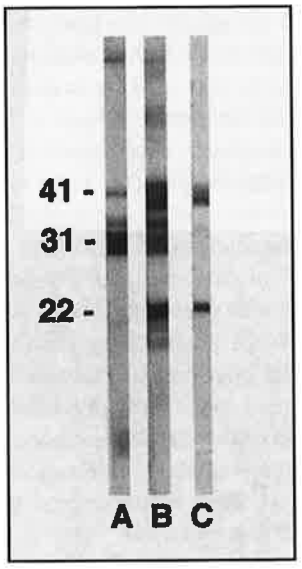

*Fig 1. IgG-*B burgdorferi *immunoblots of sera from C3H mice at 2 weeks after inoculation with different doses of viable and nonviable* B burgdorferi. *Lane A represents serum from a mouse inoculated with 10^7 heat-killed* B burgdorferi. *Note reactivity to 31 (OspA), 34 (OspB), and 41 (flagellin) kDa antigens. Lane B represents serum from a mouse inoculated with 10^7 viable* B burgdorferi. *In addition to reactivity to OspA, OspB, and flagellin, there is reactivity against 22 (OspC) and 39 (P39) kDa proteins. Lane C represents serum from a mouse inoculated with 10^1 viable* B burgdorferi, *and resembles the reactivity of serum from mice infected by tick or tissue transplant. There is reactivity to OspC, P39, and flagellin.*

agrees with studies by others.[20] In addition, only mice actively infected with viable spirochetes seroconverted to P39 and OspC, and did so regardless of infecting dose.[18,19] These experiments underscore the importance of low-dose inoculation in immunology and pathogenesis studies. Therefore, dose used to infect mice will be emphasized where it is deemed critical to interpretation of results throughout this review.

Aside from differences in dose and route of inoculation, site of intradermal inoculation of experimental mice can have significant effects upon host response. Mice inoculated in the shoulder region (where most ticks attach) developed spirochetemia, *B burgdorferi* antibody, and arthritis earlier than mice inoculated in the foot. Lymphocytes from blood and spleen of mice inoculated in the shoulder region, but not foot, had impaired proliferative responses to mitogens.[21] These results demonstrate that initial site of *B burgdorferi* inoculation is an important determinant of pathogenesis, and should be kept in mind in mouse studies. Various laboratories inoculate mice in different sites.

As will be discussed later, tick-borne infection is an important element in mouse studies, particularly in vaccine studies. It is estimated that ticks deliver <300 spirochetes,[22] but this cannot be accurately assessed and is likely to be variable. Spirochetes differ significantly in unfed compared to feeding ticks, and differ between culture and host-adapted states. For example, OspA is abundantly expressed on spirochetes in the midgut of unfed ticks,[23-25] but is rapidly lost within 24 hours of onset of feeding as they migrate from the midgut to the salivary glands.[26] A reverse shift, in which OspC is up-regulated, also appears to take place.[24,27] These events, in which spirochetes undergo change and must migrate in the feeding tick, explain why ticks must be attached for approximately 2 days for infection to be transmitted to the host, and why partially fed ticks transmitted spirochetes faster upon reattaching and feeding.[28] Once spirochetes have been transmitted to the skin of the mouse, they reside locally in the dermis for a few days before dissemination. During this period, excision of the skin around the site of tick attachment up to 2 days after detachment of the infecting tick aborted disseminated infection.[29,30] Topical treatment of the site of tick attachment with antibiotics during the first 2 days after detachment also prevented disseminated infection in mice.[31]

The humoral immune response of mice infected by ticks parallels that generated by infection of mice with low syringe doses ($<10^4$) of spirochetes.[18,20,32,33] An interesting observation was the finding that mice inoculated by syringe (2×10^7 spirochetes), by suspensions of infected ticks containing approximately 6×10^5 spirochetes, or by tick-borne infection developed equivalent *B burgdorferi* ELISA titers through 110 days, but only serum from mice infected by syringe or tick suspensions seroconverted to OspA, whereas mice infected by tick feeding did not. Furthermore, infection of ticks by feeding on mice infected with these different inocula (xenodiagnosis) indicated that ticks feeding on tick-infected mice had a higher rate of infection compared with ticks feeding upon mice initially infected by syringe or with tick suspensions.[32] This could be interpreted in several ways, but the syringe- or tick-suspension infected mice developed an OspA response, which makes spirochetes in the midgut of feeding ticks vulnerable to OspA antibody.[26] This will be discussed later in the context of vaccine immunity.

Mice can also be infected by transplantation of tissue from other infected mice. This has usually been accomplished by inserting a small piece of skin from an infected mouse into the subcutis of a recipient mouse.[18,34] This allows examination of host responses to spirochetes that have already adapted to the host. Such spirochetes are markedly different from cultured spirochetes or spirochetes in ticks. For example, host-adapted spirochetes were no longer vulnerable to OspA-induced immunity, and could disseminate and induce disease in OspA hyperimmune mice, whereas such mice were resistant to syringe-borne spirochetes[18] as well as tick-borne spirochetes.[25] Such pieces of tissue contained small numbers of host-adapted spirochetes (probably less than 600) and upon infecting the recipient, elicited a humoral immune response equivalent to low dose syringe or tick-borne exposure.[18]

There has been no evidence of contact transmission or detection of viable spirochetes in urine of laboratory mice. Lung, urinary bladder, and kidney are frequently infected, but spirochetes in these tissues are present in the

connective tissue of the serosa, subserosa, submucosa (bladder), and periarterial connective tissue (lung, kidney), rather than lumina of tubules, ureters, bladder, or airways.[3] In utero infection with *B burgdorferi* has been documented in a small number of human fetuses and newborns.[35-37] Acute infection of pregnant mice during early gestation resulted in nonspecific fetal death, but fetuses in dams chronically infected at the time of pregnancy were unaffected. Using *ospA* primers, *B burgdorferi* was detectable by PCR in the uterus of acutely infected mice, but not chronically infected mice, and was only rarely detected in fetal tissues.[38] Thus, mice pose a minimal risk of contagion among themselves or to human handlers.

Infectivity of different *B burgdorferi* isolates and strains

It is now well established that *B burgdorferi* is grouped into a genospecies complex, *B burgdorferi sensu lato*, encompassing *B burgdorferi sensu stricto*, *Borrelia afzelii, Borrelia garinii, Borrelia japonica,* and, possibly, other groups. It is suspected that members of these different groups may be associated with different disease patterns in humans.[39,40] Many isolates of *B burgdorferi sensu lato* have been obtained from ticks, animals, and humans, and they vary considerably in their ability to infect and produce disease in laboratory mice, but mice are susceptible to both infection and induction of disease with members of *B burgdorferi sensu stricto*, *B afzelii,* and *B garinii*.[41] This author has tested many isolates, finding that most induced disseminated infection and joint and heart disease in mice (unpublished observations). It has been our experience that some isolates, including some California sensu stricto isolates, infect mice, but tend to remain restricted to the skin, even after repeated mouse passage (unpublished observations), emphasizing the importance of skin for this vector-borne organism.

B burgdorferi isolates that have been maintained through multiple in vitro passages often lose their infectivity and pathogenicity,[42-45] but this is not absolute, as multiple passages through infant mice can often restore infectivity and virulence (unpublished observations). Nonclonal populations of spirochetes can contain subpopulations of spirochetes with low infectivity for mice that may overgrow or dominate more infectious organisms.[45] We have purposely passed a clonal N40 isolate with high infectivity over 100 times and it has retained its pathogenicity in mice (unpublished observations). Furthermore, low-infectivity and nonpathogenic isolates of *B burgdorferi* can be converted to high infectivity and pathogenic status by mouse passage, such as the conversion from an originally infectious, nonpathogenic *B burgdorferi* 25015[46] to an infectious, pathogenic *B burgdorferi* 25015.[47] Both infectivity and/or pathogenicity of a given *B burgdorferi* isolate can be significantly influenced by dose, route of inoculation, age of the mouse, and genotype of the mouse. Tick isolates are particularly heterogeneous, and long-term infection of mice with tick isolates of *B burgdorferi* has resulted in isolation of heterogeneous spirochetes after long-term infection, whereas initial infection with clonal spirochetes resulted in homogeneous populations of spirochetes after 12 months of infection.[4] Animal experiments should therefore be executed with clonal populations of *B burgdorferi* or defined mixes of clonal populations to obtain meaningful data relative to the host-parasite interaction. Feeding larval ticks upon mice experimentally infected with defined isolates of *B burgdorferi*, with subsequent molting of ticks into the nymphal stage, can be a useful means of infecting laboratory mice with nymphal ticks containing known isolates of *B burgdorferi*.[25,26,48,49]

Genetic susceptibility of mice to infection and disease

Host age and genotype are significant factors in outcome of experimental infection of mice with *B burgdorferi*. Both outbred and inbred mice are equally susceptible to infection, but susceptibility to disease differs significantly between genotypes. Genetically determined disease susceptibility in the mouse is age-dependent, as several diverse genotypes of mice, representing different major histocompatibility complex (MHC) haplotypes (BALB/cByJ, H-2^d; C3H/HeJ, H-2^k; C57BL/6J, H-2^b; SJL/J, H-2^s; SWR/J, H-2^q) developed uniformly severe disease when inoculated at 1 week of age, but displayed significantly different patterns of disease susceptibility when inoculated at 3 or more weeks of age. C3H and SWR mice developed significantly more severe disease than the other genotypes. In addition, C3H mice were shown to be susceptible to induction of disease at 3 days, 3 weeks, or 12 weeks of age, but disease severity was less in older mice.[1] Based upon this work, the C3H mouse has emerged as the disease-susceptible genotype of choice and the BALB or C57BL mice as disease-resistant strains.

These data imply that differences in genetically based disease susceptibility are not manifest in infant mice, which are immunodeficient, but when older mice with functional immune systems are examined, differential susceptibility becomes apparent. This has led to considerable speculation about the role of mouse immunogenetics and Lyme disease susceptibility. In one study, 17 different strains of mice were analyzed, with the general conclusion that H-$2^{b,j,s,j,k}$ strains of mice developed moderate to severe disease, whereas H-2^d mouse strains developed minimal disease,[50] but disease was assessed as gross joint swelling rather than microscopic examination of tissue (see following). Inbred and recombinant inbred mice of different H-2 haplotypes developed significantly different

immune responses to *B burgdorferi* infection, as measured by immunoblot responses, immunoglobulin isotypes, and cytokine responses.[20,32,33,50-53] This is discussed further under immune response, but the suspected relationship between MHC haplotype and disease susceptibility suggests a role of acquired immunity in Lyme disease pathogenesis.

Although MHC haplotype is likely to be linked with differential immune responses, there are other intrinsic genotype-related factors that are not necessarily linked to MHC and acquired immune response. For example, infection of genetically disease-susceptible C3H and disease-resistant BALB mice with and without severe combined immunodeficiency (*scid*) revealed that disease susceptibility of C3H mice and resistance of BALB mice was differentially expressed in the first month of infection, regardless of ability or inability to mount an acquired immune response (Fig 2).[54] Comparison between congenic mice of the same genotype but with different MHC haplotypes has also weakened the MHC-linked susceptibility theory. C3H/HeN ($H2^k$) mice were compared with congenic C3H/SW ($H2^b$) mice and B10.BR ($H2^k$) mice were compared with congenic C57BL/10 ($H2^b$) mice. Arthritis severity was equivalently severe among C3H mice and equivalently mild among C57BL mice, regardless of H2 haplotype.[53] Furthermore, the highly disease-resistant status of C57BL mice could be converted to a level of high disease susceptibility equivalent to C3H mice with a single gene mutation (*beige* mutation) that influences NK cell and leukocyte function. Depletion in mice of NK cells had no effect upon disease susceptibility, so the *beige* mutation appeared to convert mice to a disease-susceptible status through leukocyte dysfunction.[55] Thus, both innate and acquired immune responses are likely to be important determinants of genetically determined disease susceptibility in *B burgdorferi* infected mice. Acquired immunity is likely to influence ultimate severity of disease by its role in causing disease resolution (discussed in the following), rather than directly influencing susceptibility to disease induction, which occurs before acquisition of specific immunity.

Human patients with severe chronic Lyme arthritis have been reported to be associated with an increased frequency MHC class II DR4, and to a lesser extent, DR2 haplotypes. The DR4 alleles most strongly associated with susceptibility are Dw4, Dw13, and Dw14.[56] In addition, DR4+ patients with antibiotic-resistant arthritis may have an unusually strong humoral response to OspA and B, suggesting a possible role of MHC-restricted reactivity to OspA/B in the pathogenesis of chronic arthritis.[57] For these reasons, susceptibility of B10 transgenic mice with chimeric MHC class II genes in which the $\alpha 1$ and $\beta 1$ domains of human DR4Dw4 replaced the corresponding

Fig 2. BALB (left) and C3H (right) scid *mice at 30 days after infection with* B burgdorferi, *depicting genetically based differences in disease severity. Note the difference in joint swelling in the absence of an acquired immune response.*

domain of mouse $I\text{-}E^d$ was evaluated. These mice mount DR4Dw4-restricted immune responses.[58] B10 nontransgenic and B10 DR4Dw4 transgenic mice were inoculated with *B burgdorferi* and evaluated for disease and antibody response for up to 180 days. Both groups developed disease, and disease resolved in both groups. Both types of mice developed antibody responses that did not include reactivity to OspA or B. Thus, the DR4Dw4 transgene did not predispose mice to chronic arthritis or inappropriate humoral responses to OspA/B.[59]

Disease manifestations and kinetics of infection

As noted throughout this review, infection and disease are subject to a number of experimental variables, including both spirochete and host factors. Nevertheless, there is a common pattern of infection and disease in mice, and comparative studies can be based upon studies utilizing intradermal inoculation of susceptible C3H mice with low doses ($<10^4$) of relatively virulent spirochetes (N40 strain). Spirochetemia could be detected within 5 days of intradermal inoculation of C3H mice with 10^4 *B burgdorferi* N40, with subsequent isolation of spirochetes from a variety of tissues, including urinary bladder, spleen, brain, kidney, and skin. During the first 5 days of infection, spirochetes could be visualized with silver stain penetrating arterial walls (Fig 3) and invading connective tissue in and around joints. Inflammation, consisting initially of neutrophilic leukocytes and later mixed populations of leukocytes, was concomitant with the visible presence of spirochetes in these sites. There was an early tendency for spirochetes to favor attachment sites of ligaments and tendons to bone with attendant inflammation (Fig 4). Proliferative polysynovitis, rich in fibrin and neutrophils, subsequently involved many tendons, ligaments, bursae, sheaths, and joints, especially tibiotarsal joints, peaking in severity at 2 to 3 weeks after inoculation (Figs 5, 6).

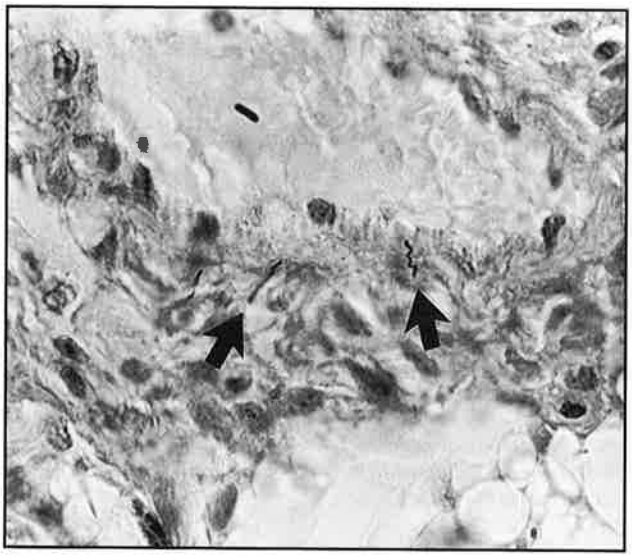

Fig 3. Spirochetes penetrating an arterial wall in tissue near a joint, 2 weeks after B burgdorferi *infection of a C3H mouse.*

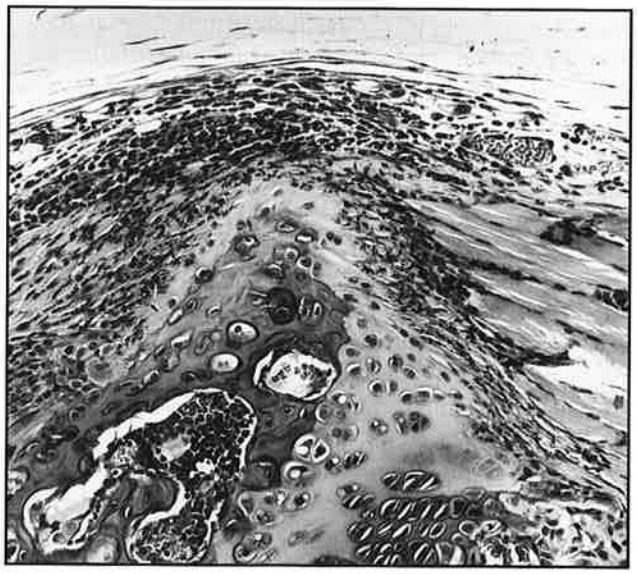

Fig 4. Tibial crest of a C3H mouse at 2 weeks after B burgdorferi *infection. There is inflammation of the connective tissue at the insertion sites of ligaments and muscle to the bone (enthesopathy).*

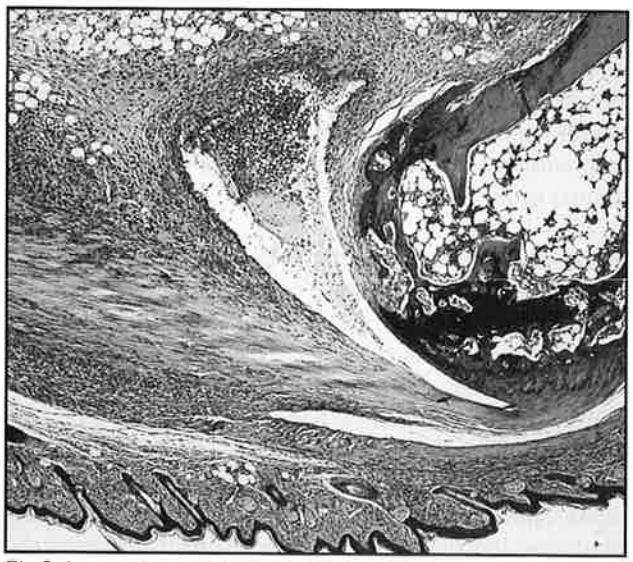

Fig 5. Acute arthritis of the tibiotarsus in a C3H mouse, 2 weeks after B burgdorferi *infection. Note severe inflammation of the Achilles tendon, perisynovial tissue, and synovium, with exudation into the joint lumen. Bone at right is the calcaneus.*

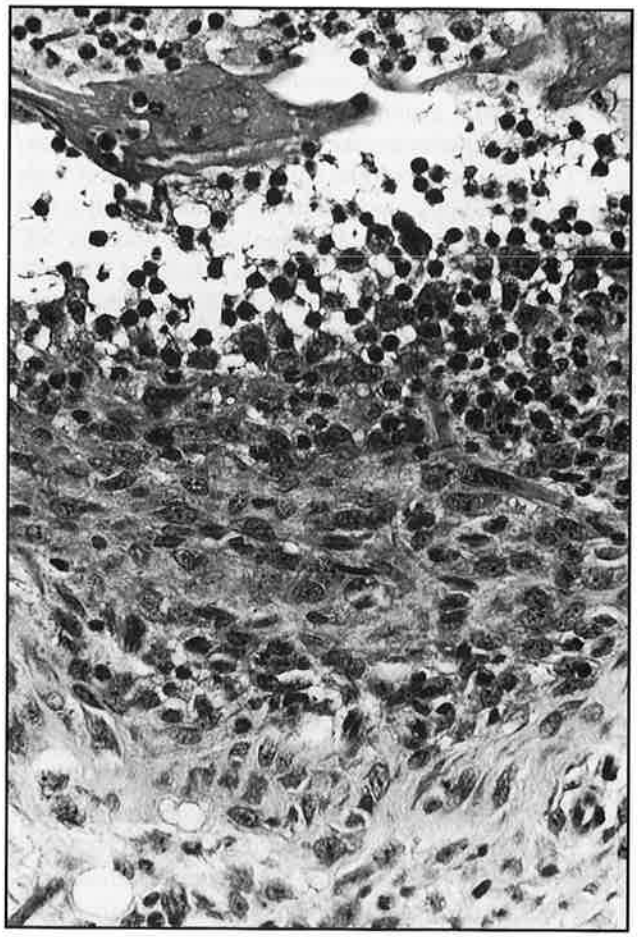

Fig 6. Tibiotarsal synovitis in a C3H mouse, 2 weeks after B burgdorferi *infection. There is marked proliferation of the synovium and exudation of neutrophilic leukocytes and fibrin into the joint lumen (top).*

Spirochetes could be visualized in the hyperplastic synovium and surrounding connective tissue, but were seldom found in the synovial lumina (Fig7).[3] There is limited human clinical material available for comparison, simply because autopsy material of acute Lyme disease is not available and synovial biopsies are generally obtained only in patients with atypically refractory or chronic disease. Nevertheless, the fibrinopurulent and proliferative features of synovitis are apparent in acute human Lyme disease[60] and material from experimentally infected

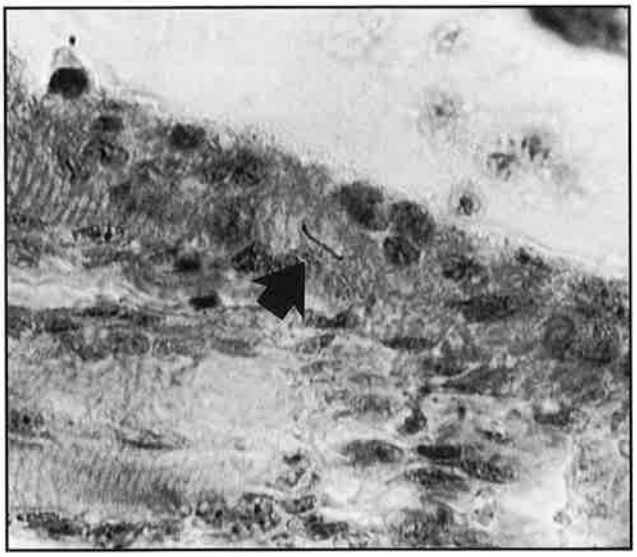

Fig 7. Spirochete within the synovium during acute arthritis in a C3H mouse. Intact organisms are seldom found in the synovial lumen.

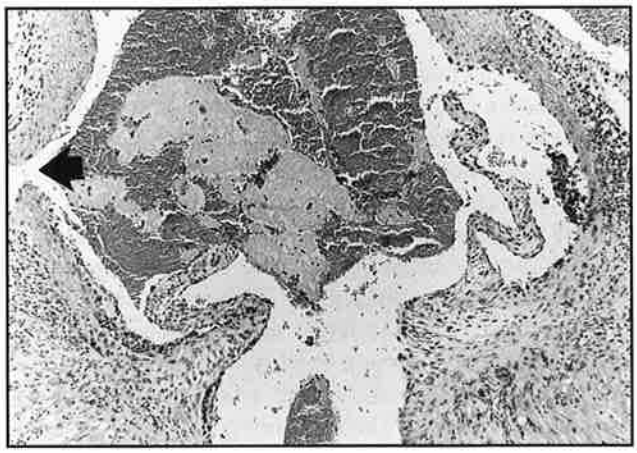

Fig 9. Aortic valve of the heart from a C3H mouse, 2 weeks after B burgdorferi *infection. There is inflammation of the aortic wall and surrounding connective tissue at the base of the heart. Note the orifice of the coronary artery at the left, which is often encompassed in the inflammation.*

Peromyscus mice,[11] rats,[5,6] hamsters,[8] rabbits,[42] dogs,[15] and monkeys.[16,17]

A remarkable lesion is the often severe periarticular edema, a feature found in the human condition, in which there is disproportionate joint swelling relative to the degree of underlying joint disease.[61] This is most easily observed in the hairless region of the tibiotarsus of the mouse at around 2 weeks after inoculation, then subsides.[1,3] Typically, the edematous tissue contains relatively large numbers of spirochetes.[3] Unfortunately, periarticular subcutaneous edema is often misconstrued as being a gross representation of arthritis, but in fact the two lesions

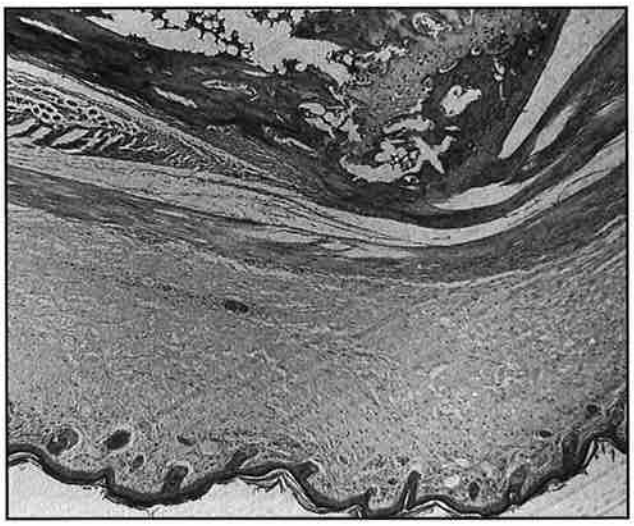

Fig 8. Severe dermal and subcutaneous edema in the tibiotarsus of a C3H mouse, 2 weeks after infection with B burgdorferi. *Grossly visible joint swelling is often due to edema, which does not necessarily correlate with underlying arthritis. In this specimen, there is no underlying arthritis in the adjacent synovial space.*

are not reflective of one another. For example, severe tibiotarsal swelling (edema) can be manifest without significant underlying arthritis (Fig 8), whereas severe arthritis is often present without edema. Although gross joint swelling and arthritis (which can only be assessed microscopically) are both useful indices for experimental evaluation, studies should (but often do not) make a careful distinction between the two phenomena.

Cardiac involvement occurs in up to 8% of patients with Lyme disease, and is associated with conduction system disturbances, pericarditis, and myocarditis.[60,62-65] In addition, there may be an association of Lyme disease with cardiomyopathy.[66-69] Limited biopsy and autopsy material has demonstrated infiltration of the myocardial interstitium, endocardium, and epicardium with mononuclear leukocytes, including macrophages, lymphocytes, and plasma cells,[60,62-65,70,71] and spirochetes have been readily demonstrable in the extracellular connective tissue.[62-64,70,71] In the C3H mouse, carditis evolved with detectable spirochete invasion of cardiac tissue within 7 days of intradermal infection. Inflammatory lesions occurred in the connective tissue of the heart, including the heart base, walls of great vessels emerging from the heart base, endocardium, epicardium, and myocardial interstitium. The region around the aortic valve and root of the aorta was consistently involved, often incorporating walls of the coronary sinus and coronary arteries (Figs 9, 10). Although mice cannot be effectively monitored for conduction defects, they manifested both bradycardia and tachycardia during peak disease.[3,4,72,73] Immunodeficient mice also developed carditis, with similar histologic fea-

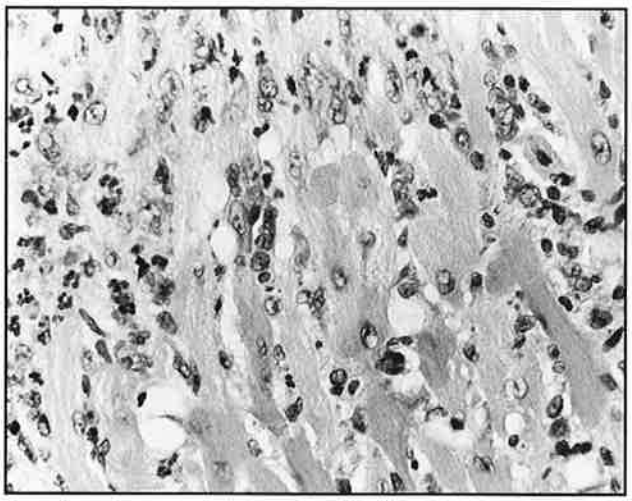

Fig 10. Nonsuppurative myocarditis in the heart from a C3H mouse, 2 weeks after B burgdorferi *infection. The myocardial interstitium is infiltrated with mononuclear leukocytes with no myofiber necrosis.*

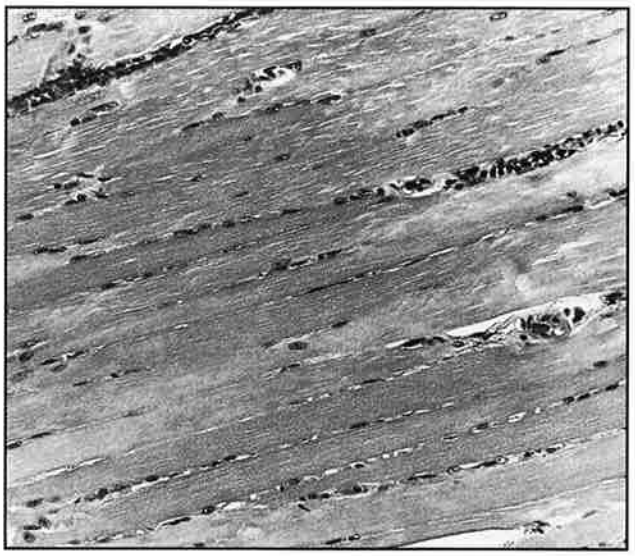

Fig 11. Nonsuppurative myositis in the leg muscle of a C3H mouse, 2 weeks after B burgdorferi *infection. The interstitium is infiltrated with mononuclear leukocytes with no myofiber necrosis.*

tures.[74-77] Studies in both immunodeficient and immunocompetent strains of mice have confirmed that macrophages are a predominant cell type in murine carditis.[73,75,76] In both immunocompetent and immunodeficient mice, spirochetes were plentiful in the early weeks of infection, and were demonstrated by silver stain, immunohistochemistry or nucleic acid in situ hybridization in the connective tissue of the heart base, endocardium, epicardium, and myocardium.[3,4,73-75] In one study, spirochetes were found by electron microscopy to be both extracellular as well as within cardiac myofibers in the early weeks of infection.[78]

During the first few weeks of infection, in which spirochetes are disseminating and disease is evolving, quantitative PCR has indicated that disease-susceptible C3H mice supported larger numbers of spirochetes in various tissues, including joints, compared with disease-resistant BALB mice. Spirochetes were detectable earlier, were present in larger numbers, and were cleared from several tissues later in C3H mice compared to BALB mice. There was a five- to ten-fold difference in spirochete numbers in various tissues between these genotypes during peak infection.[79] Results of PCR, using both plasmid and chromosomal targets, correlated well with culture results, and PCR-detectable nucleic acids disappeared rapidly following antibiotic treatment of mice.[3,80] Notably, there was an imbalance of excessive spirochete plasmid target relative to chromosomal target in mouse tissues, but not in cultured spirochetes,[18] similar to findings in human Lyme disease patients.[81] Analysis of spirochete presence by culture and PCR and relative numbers by in situ hybridization, immunohistochemistry, and silver stains has revealed that spirochetes invaded cardiac tissue of disease susceptible C3H mice earlier, were present in larger numbers, and were cleared from cardiac tissue later compared to disease-resistant C57BL mice.[72] Interference with leukocyte function with the *beige* mutation in normally disease-resistant C57BL mice resulted in detectably larger numbers of spirochetes in their diseased joints, resulting in more severe arthritis.[55] Thus, these studies suggest that early, innate host responses are likely to determine the number of spirochetes in target tissues, which in turn is directly reflective of disease severity.

Myositis has been described as a rare complication of human Lyme disease. There is perimysial and epimysial infiltration of mononuclear leukocytes, shown to be principally macrophages and CD4+ lymphocytes, without myofiber necrosis.[60,82-84] Inflammation of skeletal muscle was found to variable degrees in different genotypes of *scid*, athymic, and euthymic mice infected with *B burgdorferi*. Infiltration of muscle connective tissue and perivascular connective tissue with mononuclear leukocytes was apparent primarily in muscle adjacent to joints, but was also found elsewhere. The principal infiltrating cells were macrophages and lymphocytes and myofiber necrosis was minimal.[85] Nonsuppurative myositis was also described in triple deficient mice with numerous spirochetes visible within the interstitial connective tissue of muscle,[74] which was also the case in the early stages of infection of C3H mice (Fig 11).[3] There was a pronounced predilection of spirochetes to invade and colonize connective tissue at the attachment sites of muscle, tendons, and ligaments to bone, with spirochetes being visible in large numbers in these sites during the early phases of infection

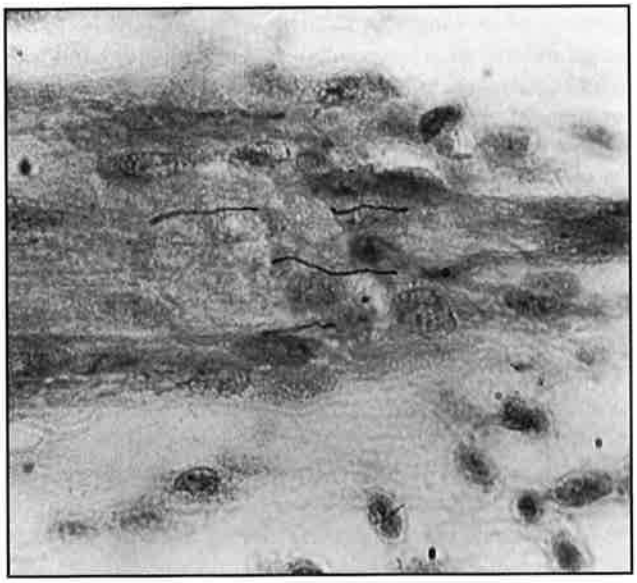

Fig 12. Spirochetes in connective tissue of the insertion site of muscle to bone in a C3H mouse, 2 weeks after B burgdorferi *infection. Spirochetes are commonly found in these locations.*

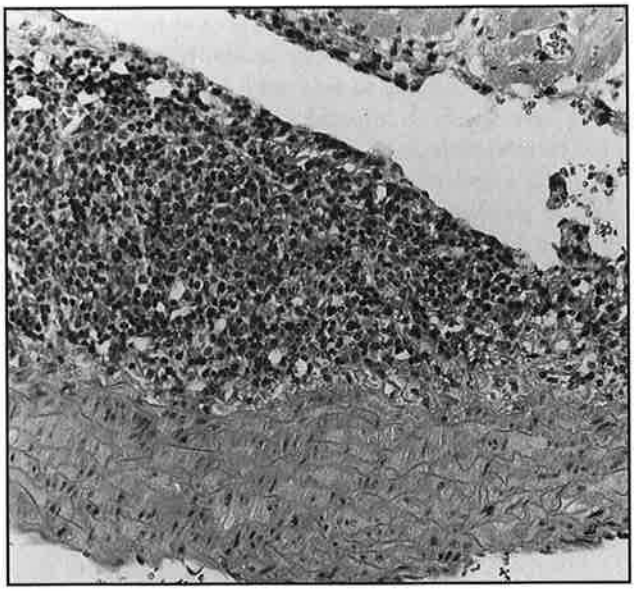

Fig 13. Segmental infiltration of the arterial adventitia with lymphocytes and plasma cells at 90 days after infection of a C3H mouse with B burgdorferi. *These segmental infiltrates are common around the aorta, at the base of the heart, and also peripheral arteries during chronic infection.*

(Fig 12).[3]

Vasculopathy, with intimal proliferation and obliteration in association with discernable spirochetes in the lesions, has been described as a feature in synovial biopsies from human Lyme disease patients.[60,86] In addition to the vascular lesions involving the root of the aorta and coronary arteries, C3H and BALB mice were found to develop segmental arteritis in peripheral arteries, particularly ramifications of the saphenous arteries. During early and recurrent bouts of disease, lesions consisted of transmural inflammation, with margination of leukocytes on the endothelium, and infiltration of the muscle. Typically, these sites were segmental, and had concomitant intense lymphoplasmacytic infiltrates in the adventitia. In mice with quiescent disease, segmental periarterial infiltrates of lymphocytes and plasma cells were often found (Fig 13) and these lesions tended to contain visible spirochetes at their periphery.[4] A proliferative lesion, possibly reminiscent of the proliferative vasculopathy noted in humans, has also been found in the early stages of infection of C3H mice, in which there was profound intimal proliferation of segments of the saphenous artery[3] and coronary vessels (Fig 14). It was very apparent that spirochetes preferentially invaded arterial walls (rather than venous) during their egress into joint tissues in the early phases of infection.[3] In addition, chronically infected immunocompetent mice developed perivascular infiltrates of mononuclear leukocytes around small arteries in a number of tissues, particularly submucosa of the urinary bladder.[4,87,88]

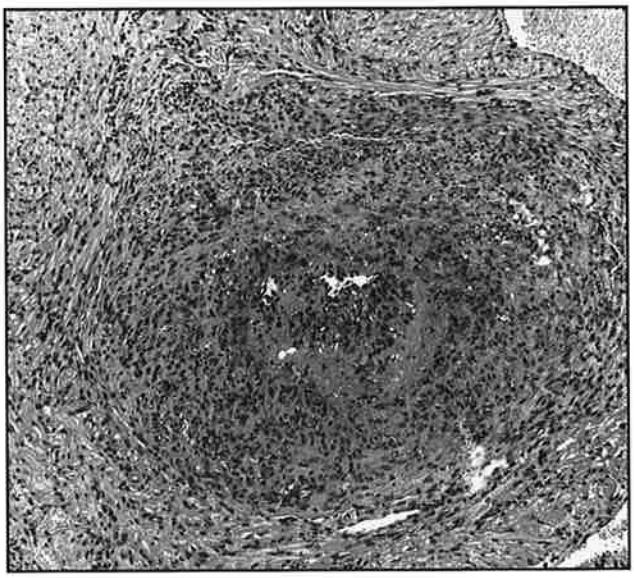

Fig 14. Acute proliferative panarteritis of the coronary artery of a C3H mouse at 2 weeks after B burgdorferi *infection. Proliferative arteritis is common in the coronary and peripheral arteries during acute infection.*

Humans develop a number of peripheral and central neurologic manifestations, which are reviewed elsewhere,[89] but neurologic disease has not been found in the mouse. Spirochetes could be cultured from the brain during the early weeks of infection in *scid* mice.[54,90] Their presence in the brain during this period was probably a reflection of concomitant spirochetemia. We have examined brain, spinal cord, and peripheral nerves for inflammatory lesions, and although there may be occasional

mild meningeal or perineural leukocyte infiltrates, they are inconsistent and relatively insignificant. Brain infection has been induced in *scid* mice with *B hermsii*[91]; *scid* mice have also been infected with *B turicatae* and 2 variable membrane protein serotypes (A and B) were isolated from the mice. Inoculation of mice with clonal populations of serotype A or B revealed that only the A serotype invaded the brain.[92] Although of interest to Borrelia biology, there is no reason to believe that these agents serve as close parallel models for Lyme borreliosis.

There is a compelling association of neurologic disease in humans with the development of an antibody response against an antigen in neural tissue that shares reactivity with an epitope on the flagellin protein of *B burgdorferi*.[93,94] To investigate this correlation further, C3H transgenic mice were created that expressed the flagellin epitope (amino acids 213-224) that binds antibodies putatively involved in human disease. The transgenic mice expressed flagellin epitope selectively in the nervous system, as part of a fusion protein with myelin basic protein. Both transgenic and nontransgenic mice developed antibodies to flagellin, including the incriminated epitope, but there was no evidence of neurologic disease or other alterations in the course of infection or disease.[95]

Persistent infection and disease

A feature of human Lyme disease, recognized prior to knowing its etiology and treatment with antibiotics, is spontaneous resolution of disease without benefit of treatment, including erythema migrans, cardiac manifestations, neurologic symptoms, and musculoskeletal symptoms.[61] These events are not due to recovery from infection, as there is mounting evidence that many patients remain persistently infected for months or years.[68,96-112] Disease resolution is no doubt driven by host immune response, which for unknown reasons is incapable of completely eliminating spirochetes, with episodic recurrence of disease.

The mouse model follows a similar course of acute disease, followed by disease resolution with episodic recurrence over the course of persistent infection. Inflammation in joints, hearts, and vessels subsided dramatically beginning around 3 to 4 weeks after intradermal inoculation of both C3H and BALB mice. By 60 to 90 days, there was virtually no active inflammation in any joint, and hearts and peripheral vessels had segmental lymphoplasmacytic infiltrates in their adventitia, but no endo- or transmural inflammation typical of early stages of infection. Spirochetes could no longer be visualized in joint tissue and only small numbers were found in heart and vessel lesions. During this stage of infection, the number of visible spirochetes in tissue was very small, spirochetemia ceased (at around 3 weeks), and the frequency of isolation from urinary bladder, spleen, and other internal organs declined.[3,4,72] On the other hand, skin was consistently infected at all stages of infection, starting at about 10 days after inoculation (in sites distant from the site of inoculation).[3] Spirochetes were detected in the skin of virtually all mice by culture or PCR for 1 or more years after infection.[4] When groups of both disease-susceptible C3H and resistant BALB mice were examined at 6 and 12 months of infection, approximately 10% of the mice were spirochetemic or had active arthritis or carditis. Arthritic lesions were usually less severe and involved fewer joints than during the initial weeks of infection, but both joint and heart lesions had microscopic features of recurrent, acute disease. Spirochetes were again visible in these actively inflamed tissues.[4] These observations suggest that mice, like humans, develop intermittent episodes of recurrent spirochetemia and disease during the course of persistent infection.

The clearest evidence for the role acquired immunity in Lyme disease resolution has been derived from studies utilizing *scid* mice. Such studies indicated that evolution of disease occurred in the absence of acquired immunity and that disease (arthritis and carditis) resolution requires an acquired immune response. These are fundamentally important observations in understanding the pathogenesis of Lyme disease. In the absence of acquired immunity, *scid* mice developed progressively severe arthritis, with virtual pannus of joint lumina and erosion of articular cartilage and bone with exuberant proliferation of synovium (Fig 15).[54,76,77] Spirochetes were abundant in this proliferating synovium, yet were not found in such numbers in other tissues, indicating that joint tissue is truly a site of targeted preference for this pathogen. Heart lesions did not become progressively severe, but remained active, rather than resolved, and spirochetes remained plentiful.[54] Thus, disease resolution requires acquired immunity.

Mechanisms of spirochete persistence

As noted previously, persistent infection in both humans and mice, as well as a variety of other animals, is a common, if not predictable phenomenon in Lyme disease. This has led to speculation about a number of potential mechanisms for spirochete persistence and evasion of host immunity, including persistence in immunologically privileged sites such as intracellular sequestration and antigenic modulation, analogous to *B hermsii*, a cause of relapsing fever.

Intracellular localization is an attractive hypothesis for evasion of immune clearance. In vitro studies have demonstrated intracellular localization of spirochetes in macrophages, fibroblasts, and endothelial cells.[113-116] As tempting as these observations may be, they do not reflect existence within the immunologically responsive host,

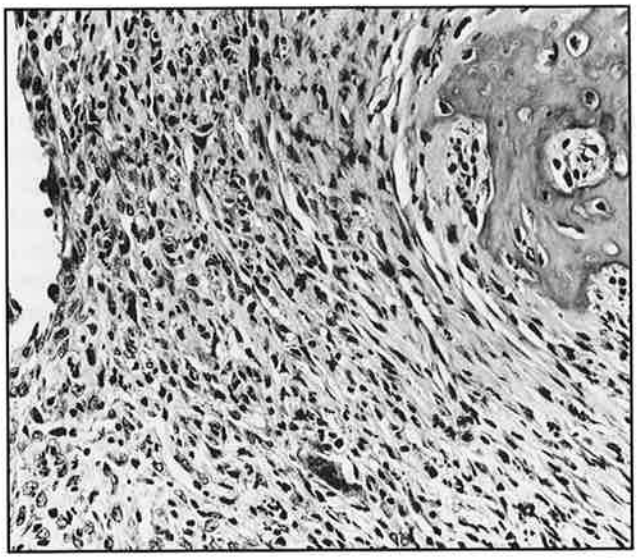

*Fig 15. Tibiotarsus of a C3H-*scid *mouse at 60 days after* B burgdorferi *infection. Normally, arthritis is resolved at this interval in immunocompetent mice, but in* scid *mice there is exuberant proliferation of synovium, resulting in occlusion of joint lumina (left) and loss of articular cartilage from bone (right).*

Fig 16. Spirochete in skin of a C3H mouse, 1 year after B burgdorferi *inoculation (of a distant site). Spirochetes can be consistently found in the dermal connective tissue of persistently infected mice. They appear extracellular and do not incite local inflammation.*

which is far more difficult to evaluate. For example, fibroblasts grown in vitro had intracellular localization of *B burgdorferi*, protecting them from ceftriaxone treatment,[116] but mice treated with antibiotics, including ceftriaxone, at different stages of infection were readily cured of infection.[34,80,117] Mouse studies have shown that during persistent infection (1 year or more of infection), *B burgdorferi* was consistently detected in skin by culture and PCR. Microscopic analysis of skin from mice infected for 1 year revealed rare, fully elongated, extracellular organisms in all mice examined (Fig 16). Spirochetes were most commonly seen in the papillary dermis, a logical site for a tick-borne pathogen.[4] During the early phases of infection, when spirochetes were far more numerous, they could be readily identified in joints, skin, vessels, hearts, kidney, urinary bladder, spleen, etc., with a very clear predilection for connective tissue.[3,4,72] Indeed, the experienced pathologist can predict the location and distribution of spirochetes in tissues. In triple immunodeficient mice, spirochetes were also identified in the connective tissue of the bowel wall.[74] In all of these studies, spirochetes appeared to be extracellular. With immunohistochemistry, fragments of spirochetes could be visualized in the cytoplasm of macrophages in active lesions, but intact spirochetes were extracellular.[18,19,72] An exception to these general observations was the finding of spirochetes by electron microscopy within the sarcoplasm of cardiac myocytes in the early weeks of infection in mice.[78] In spite of finding some intracellular forms of organisms during the early stages of infection when spirochetes are plentiful, a far more compelling observation is the consistent visualization of fully elongated, extracellular organisms in tissues of persistently infected hosts. Clinical specimens from humans, in which spirochetes have been visualized, have revealed apparently extracellular forms, analogous to findings in the mouse.[60]

Antigenic modulation is another attractive explanation for spirochete persistence and might explain the recurrent nature of Lyme disease. Precedent has been set with *B hermsii*, which effects major shifts in its variable membrane proteins through purposeful gene rearrangement, so that recurrent episodes of spirochetemia represent antigenically modified populations of spirochetes that are unfamiliar to the immune system.[118] There is considerable genetic and antigenic variation among isolates and genospecies of *B burgdorferi*,[119-121] and variation could be induced following extended in vitro passage.[43,44] Growth of *B burgdorferi* in the presence of OspA or B antibody induced antibody-resistant variants that lacked specific Osp expression, had *osp* gene mutations, or lost entire *osp* genes.[122,123] Recombination between homologous OspA/B-encoding genes, with deletions and chimeric gene fusions, have also been demonstrated, with resultant variation of Osp expression.[121] In favor of the *B hermsii* analogy, *B burgdorferi* OspC shares similarities with variable membrane proteins of *B hermsii*.[124-127] OspA and B variants of *B burgdorferi* have been isolated from OspA/B-immunized mice[128,129] and sequential samples from the same patient.[130] This must be interpreted with caution, however, as *B burgdorferi* isolates contain nonclonal populations of

spirochetes. For example, OspB is very polymorphic among clones derived from uncloned *B burgdorferi* isolates,[131] which can have significant influence upon infection of the OspB-immunized host.[128,129]

Studies in mice have indicated that *B burgdorferi* persistence does not require either antigenic or genetic variation, although such events may coincidentally occur. Considerable protein variation was found among spirochetes isolated from mice infected for 1 year with uncloned *B burgdorferi*[4,132] but no heterogeneity was found among 1 year isolates following initial inoculation with clonal spirochetes.[4] OspA/B variants could be purposely selected in Osp A or B vaccinated mice infected with uncloned *B burgdorferi*.[128,129] There was no detectable antigenic discrimination by serum from early or late stage infection against clonal homologous early or late isolates of *B burgdorferi*, and actively immune mice, cured of infection with antibiotic, resisted challenge equally with their own early and late *B burgdorferi* isolates or autograft challenge with their own infected tissue.[19,34] Spirochetes isolated from persistently infected mice in which there were no active disease manifestations were fully infectious and pathogenic to naive mice, indicating that spirochetes had not lost their virulence.[4,34] There was no detectable genetic variation among 1 year isolates from mice infected with clonal spirochetes, based upon genomic macrorestriction analysis or fine sequence variation of *ospA*, *ospB,* or *ospC* genes. Although one isolate was missing the *ospD*-containing plasmid, others were apparently unmodified.[133,134] Likewise, no *ospA* or *ospB* fine sequence variation was noted among *B burgdorferi* spirochetes obtained several weeks after infection of subcutaneous chambers with *B burgdorferi* Sh-2 in either C3H or BALB mice.[135] Thus, although *B burgdorferi* is naturally antigenically and genetically variable, variation can be induced in vitro, and variants can be isolated from mice infected with uncloned spirochetes in vivo, these events do not seem to be required for successful persistence of *B burgdorferi* in the host.

Immune response

Host immune response is a critical element in Lyme disease pathogenesis and diagnosis, and there is much uncharted territory ahead. The antibody response to *B burgdorferi* in untreated patients develops gradually over the course of months to years with reactivity to an expanding array of proteins.[136-142] A consistent finding in serologic studies is a relatively strong antibody response to 22, 39, and/or 41 kDa proteins, both in the early and late stages of infection and often involving IgM.[136-143] The 41 kDa protein (flagellin) elicits a frequent and early antibody response, but is cross-reactive with other bacteria.[136-142] A highly conserved and specific 39 kDa (P39) lipoprotein elicits an early and persistent antibody response, but reactivity to this protein may be obscured or confused with reactivity to closely migrating flagellin.[136,137,139,142,144,145] One or more ca. 22 kDa proteins are recognized by serum from patients with early and late Lyme disease, including OspC,[138,146-148] and other distinct ca. 22 kDa proteins elicit antibody responses in humans.[149-156] The 31 and 34 kDa OspA and OspB are major lipoproteins, but antibodies to OspA/B are often absent, or appear only late in the course of infection.[136-142] Low levels of predominantly IgM antibody to OspA, often complexed with antigen, can be detected transiently in early infection.[157,158] In patients with chronic Lyme disease, OspA/B antibody reactivity has been noted to evolve near the beginning of prolonged episodes or arthritis in previously OspA/B seronegative patients.[57] Among many other potentially important proteins that elicit an antibody response during infection are the 60-, 66-, and 68- kDa proteins, which are homologs of the GroEL family of heat shock proteins; 71- and 73-kDa proteins, which are homologs of the DnaK family of heat shock proteins[159-162]; and 83 to 100 kDa proteins (P93), a core protein of *B burgdorferi*.[163]

As previously discussed, the murine antibody response, following tick-borne, infected tissue transplant or low dose syringe inoculation, follows the same pattern, with minimal OspA reactivity until several months into infection, and early strong recognition of flagellin, P39, and OspC.[18-20,33] As in humans, there is expanding immunoblot reactivity to a wide repertoire of *B burgdorferi* proteins over the course of persistent infection, with rising ELISA (whole *B burgdorferi* antigen) antibody titers.[4] Both C3H and BALB mice developed significant sustained elevation of serum IgM and IgG during infection with *B burgdorferi*, representing antibody directed against both *B burgdorferi* as well as unrelated antigens. IgG1, IgG2a, IgG2b, and IgG3 isotypes were elevated in infected mice, and IgG2a was markedly elevated in C3H mice compared to BALB mice, suggesting a polarized Th1-dominated response in C3H mice. In addition, this study found generalized lymphadenomegaly, with increased lymphocyte populations in both lymph nodes and spleens of infected C3H mice.[53] A number of other studies have examined serum antibody titers to *B burgdorferi*, IgG isotype responses, and differential responses to *B burgdorferi* antigens among different genotypes, haplotypes, or allotypes of mice, and although there are distinctly different patterns of response, no consistent association could be found between antibody response and disease susceptibility.[4,20,33,50,55,164] Nevertheless, the serum antibody response is critically important as an effector of protective immunity, induction of disease resolution, and maintenance of the host-parasite balance during persistent infection, which will be dis-

cussed in the following.

The human cellular immune response to *B burgdorferi* is less well understood, as studies have been performed with unpurified cell populations against whole *B burgdorferi*. Peripheral blood lymphocytes from Lyme disease patients respond to *B burgdorferi*, but lymphocytes from uninfected control individuals have also been found to proliferate nonspecifically in response to *B burgdorferi*. This nonspecific mitogenic effect was localized to B cells[165-167] and is likely to interfere with proliferative assays using impure cell populations and *B burgdorferi* as antigen. It may explain the hyperactive B cells, elevated immunoglobulin levels, lymphadenopathy, impaired NK cell function, and delayed development of humoral immunity (including seronegativity) in Lyme disease patients.[168-170]

A frequent error in interpretation has been to equate cellular responses of unpurified peripheral blood lymphocytes with cell-mediated immunity, and by extension, T cell responses. Analysis of lymphocyte mitogenic responses in mice have shown that *B burgdorferi*, extracellular blebs of *B burgdorferi*, OspA, OspB, and other lipoproteins, but not flagellin, elicited strong nonspecific, dose-dependent B cell mitogenic responses and nonspecific stimulation of immunoglobulin synthesis in naive mice that had never been exposed to the spirochete. The B cell mitogenic effect of *B burgdorferi* was quite potent, exceeding that induced by *Escherichia coli* mitogenic lipoproteins, and could function as an adjuvant for responses to other antigens. The effect was not due to lipopolysaccharide (LPS) and could be induced with multiple isolates of *B burgdorferi* and in a variety of mouse genotypes (and haplotypes).[53,164,171-178] The kinetics of this nonspecific B cell response indicated peak activity at 48 hours of culture, with decline to normal levels by 120 hours, whereas specific T cell responses, if detectable, peaked around 96 hours.[175,176] Thus, T cell responses should be evaluated at the appropriate interval, as early intervals are likely to be influenced by nonspecific B cell mitogenic effects, but later intervals are potentially complicated by growth of contaminating B cells as culture time increases.

Attempts to detect specific T cell responses to *B burgdorferi* or its antigens in mice have been variable, but indicate a relatively weak T cell response during infection, especially following low-dose inoculation, which would parallel natural infection. T cell enriched splenocytes from C3H and BALB mice actively infected after initial low dose (10^4 spirochetes) inoculation were evaluated for specific proliferative responses to *B burgdorferi* at intervals over the course of 1 year. Specific T cell responses (3 SD above control responses) were detected in both genotypes of mice, but they were inconsistent and intermittent, suggesting relatively weak T cell responses.[176] Others[87] found significantly elevated *B burgdorferi*-stimulated proliferative responses at 96 hours of culture of nylon wool-purified (T cell enriched) lymph node cells, and to a lesser extent splenocytes, from C57BL mice at 100 days after intravenous inoculation with high doses (10^7) of viable spirochetes or subcutaneous inoculation with 10^6 dead organisms.[87] Infection of BALB mice subcutaneously with 10^7 to 10^8 spirochetes, or footpad immunization with killed organisms, revealed that *B burgdorferi*-stimulated T cell responses resulted in IL-2 and IFN-γ production, but not IL-4 or IL-5, implying a Th1-dominated T cell response.[164] Delayed type hypersensitivity (DTH) reactions have been induced in mice inoculated with large numbers of viable and killed spirochetes, and could be enhanced with fewer spirochetes plus adjuvant, but could not be detected when mice were infected with 10^6 or fewer viable spirochetes in C57BL/6 ($H2^b$), AKR ($H2^k$), B10.D2 ($H2^d$), B10.BR ($H2^K$), and B10.S ($H2^s$) mice. BALB ($H2^d$) mice developed only marginal DTH responses to high doses of antigen.[87] Likewise, no DTH reactions could be detected in C3H or BALB mice actively infected with low doses (10^4) of spirochetes.[176,179] These studies underscore the importance of immunizing dose of spirochetes when evaluating cellular immune responses.

During the acute phase of *B burgdorferi* infection of both disease-susceptible (C3H) and disease-resistant (BALB) mice, there was impaired proliferation of lymphocytes to both T and B cell mitogens as well as impaired IL-2 and, to a lesser extent, IL-4 production, for up to 30 days after intradermal infection with 10^4 spirochetes. Impaired lymphocyte proliferative response was not due to diminished numbers of T or B cells. Lymph node cells responded better to concanavalin A than did splenocytes, and splenocytes responded better than lymph node cells to LPS and *B burgdorferi*. At least part of the T cell suppression could be attributed partially to prostaglandin production, as indomethacin significantly but not completely restored proliferative responses of T cells from infected mice to control levels. IL-2 production by splenocytes was more impaired than IL-4 production, suggesting that the effect, like that induced by prostaglandin, exerted a greater inhibitory effect on Th1 than on Th2 cells.[176]

B burgdorferi and its lipoproteins have been shown to induce a number of cytokines likely to affect pathogenesis. *B burgdorferi* lipoproteins, including OspA, induced TNFα in murine macrophages,[180] with lower levels induced in C3H macrophages compared to BALB macrophages.[173] Both *B burgdorferi* and TNFα-induced prostaglandin production by murine macrophages, and TNFα was found to be elevated in sera of *B burgdorferi*-infected mice.[181] Furthermore, *B burgdorferi*-induced IL-1

in human and murine macrophages.[182] Thus, either TNFα or IL-1, induced by *B burgdorferi* or its lipoproteins, can in turn stimulate prostaglandin production. Another cytokine of potential significance to pathogenesis and immune response is IL-6, which has been found to be persistently elevated, but peaking at 2 to 3 weeks (peak disease), in serum of *B burgdorferi* infected C3H mice, and much lower levels were found in infected BALB mice.[53] *B burgdorferi*, OspA, and OspB stimulated splenocytes as well as bone marrow macrophages from naive C3H and BALB mice to produce IL-6.[172,173] Much remains to be learned about the complex cytokine network, differences between mouse genotypes, and role in Lyme disease pathogenesis.

There is mounting evidence that T cells function principally as helper cells that modulate B cell responses, rather than playing a direct effector role in host immunity to *B burgdorferi* infection. Selective depletion of disease-resistant BALB and disease-susceptible C3H mice of CD4+ or CD8+ T cells with monoclonal antibodies had significant effects upon disease expression, numbers of spirochetes, and antibody responses of mice infected subcutaneously at the base of the tail with high but undefined doses of spirochetes. These studies measured gross joint swelling as an index of disease and quantified spirochetes in tissues by measuring the outgrowth of spirochetes from joint tissues placed in BSK II medium, but nevertheless demonstrated that CD4+ T cell depletion increased severity of joint swelling and outgrowth of spirochetes in both genotypes of mice, whereas CD8+ T cell depletion appeared to enhance susceptibility. Furthermore, β2-microglobulin-deficient C3H mice, which lack CD8+ cells, were found to be more resistant to disease than C3H mice.[51,183] These findings imply an important role of CD4+ T cells in disease resistance mechanisms induced by acquired immune responses (in contrast to innate immune responses). They also imply a role of CD8+ T cells in disease susceptibility, but this could also be an indirect effect resulting in altered CD4+ T cell responses.

The relative role of CD4+ Th1 or Th2 subset responses has also been examined in C3H and BALB mice. Cytokine responses were evaluated in popliteal lymph nodes from mice inoculated in the footpad with 10^5 spirochetes and gross joint swelling was assessed as an index of disease severity. BALB mice developed a Th2 pattern of cytokine secretion by lymph node cells, with high IL-4 and low IFN-γ production following *B burgdorferi* stimulation, while C3H mice developed a Th1 pattern of cytokine secretion with absent or low levels of IL-4 and high levels of IFN-γ. These effects were not found with lymph node cells from the contralateral limb or spleens.[52] Treatment of C3H mice with anti-IFN-γ or BALB mice with anti-IL-4 monoclonal antibodies reversed the disease phenotype (joint swelling). Of interest are the parallel observations that *B burgdorferi*-infected (10^6 dose) C3H mice developed more elevated IgG2a levels compared with BALB mice[53] and C3H lymphocytes produced IFN-γ in response to *B burgdorferi* stimulation,[173] indicative of a Th1-dominated response in C3H mice.

In another study, mice were inoculated with 10^5 spirochetes at the base of the tail, and splenocytes from C3H mice were shown to produce higher levels of IL-2 and IFN-γ and lower levels of IL-4 than splenocytes from BALB mice. Serum from the C3H mice had higher levels of *B burgdorferi* specific IgG2a and lower IgG1 compared to serum from BALB mice. Treatment of either C3H or BALB mice with IL-4 antibody resulted in greater joint swelling and higher numbers of spirochetes growing from joint tissue, whereas treatment of mice with IFN-γ antibody reduced joint swelling and spirochete outgrowth.[183]

Others,[164,184] using BALB mice infected with 10^7 to 10^8 spirochetes, or immunized with killed spirochetes, have reported exclusively Th1 responses by splenocytes and lymph node cells, characterized by IL-2 and IFN-γ secretion in response to *B burgdorferi* antigen. In humans with chronic Lyme disease, T cell clones derived from synovium or peripheral blood were found to be exclusively CD4+[185-187] and those characterized to date exhibited exclusively Th1 phenotypes.[186,187] A CD4+, Th2 T cell clone was isolated from a *B burgdorferi* hyperimmunized BALB mouse and found to confer protection against 10^8 spirochetes by adoptive transfer of 10^6 cloned cells at the time of challenge. The clone was reactive against an unidentified 21 kDa *B burgdorferi* protein.[184]

Because IL-12 is induced by *B burgdorferi* lipoproteins[188] and causes IFN-γ production, it may induce a polarized Th1 CD4+ response, as well as augment NK cell cytotoxicity and cytotoxic T cell activity, the effect of anti-IL-12 treatment of C3H mice on infection (10^4 spirochetes) and disease was evaluated. Treatment resulted in a decrease in both IFN-γ and *B burgdorferi* IgG2b levels in serum, indicative of a diminished Th1 response, but mice had IgG1 and IgG2b levels equivalent to control mice, indicating no Th2 augmentation. Treatment induced a slight reduction in arthritis severity and increased numbers of spirochetes in ear tissue, based upon quantitative PCR.[189] Thus, polarized helper T cell subset responses may be involved in differential disease susceptibility among different genotypes of mice, and possibly different humans. As discussed above, acquired immune responses are only a partial explanation for differential disease susceptibility patterns among genotypes of mice, as innate immunity also plays an important role in the evolution of disease and its severity.

Adoptive and passive transfer of immunity

Transfer of immune cells or serum from infected mice to naive mice has allowed investigation into the relative contribution of the cellular and humoral arms of acquired immunity in host responses to invading spirochetes as well as existing infection and disease course. Adoptive transfer of T cell-enriched lymphocytes from infected immunocompetent mice to *scid* mice did not protect against challenge inoculation, whereas transfer of T and B cells or B cells alone were protective.[176,179] On the other hand, transfer of immune serum to *scid* or immunocompetent mice conferred strong protection against challenge inoculation. Such studies require careful attention to infecting dose when generating immune serum. As discussed previously, high-dose exposure results in an atypically strong OspA/B response and antibody directed against OspA/B is protective against syringe- and tick-borne challenge inoculation (see following). When serum from low-dose infected mice, analogous to naturally infected mice, is transferred to naive mice, it is protective, implying that antigens other than OspA/B are eliciting protective immunity.[19,34] Serum derived from mice infected by tick-borne exposure, high dose syringe inoculation, or low dose syringe inoculation all had equivalent passive protective activity in *scid* mice, even though only the serum from mice infected with high doses of spirochetes contained antibody directed against OspA.[20] Serum from naturally infected humans as well as dogs will also confer protection to passively immunized mice.[190,191] Using serum collected sequentially from low dose infected mice over the course of 1 year of infection, ELISA IgG titers to *B burgdorferi* antigen rose progressively over time, whereas passive protective antibody titers peaked at 30 days, then declined over time.[19] The peak in protective activity (30 days) in immune serum appeared to correspond with the period in which disease was undergoing regression, and the decline in titer suggests diminished antigen stimulation, as would occur following clearance of all but a few spirochetes from the host. Those spirochetes that remain elicit some degree of stimulation, as both the passive immunizing and ELISA curves decline more precipitously in mice treated with antibiotics (unpublished observation).

The above studies indicate the importance of antibody, and not T cells, in directly effecting protective immunity, but helper T cells are likely to be critical in augmenting antibody responses. In addition, antibody is also important in immunity to spirochetes after infection is established, which results in resolution of disease, but not complete clearance of infection. Adoptive transfer of T-enriched cells from both *B burgdorferi* infected (immune) and uninfected (control) immunocompetent mice to *scid* mice at 14 days of infection, an interval at which arthritis was well established, had no short-term significant effect upon infection or disease. Arthritis eventually began to resolve in *scid* mice receiving T cells transferred from both immune and non-immune donor mice, but this effect could not be attributed to T cells alone, as the mice developed antibody to *B burgdorferi*, indicating reconstitution of B cell activity. On the other hand, passive transfer of immune serum from low-dose infected (10^2 spirochetes intradermally), immunocompetent mice to *scid* mice had strong protective, postinfection curative and disease-modulating effects. Groups of *scid* mice were treated with a single injection of 90 day immune serum or normal mouse serum at -18 hours, or multiple injections on days 4, 8 and 12 or days 12, 16, 20, 24, and 28 relative to inoculation with 10^4 spirochetes.[192] The -18 hour interval for treatment was chosen because of the established effectiveness of passive transfer of immune serum at -18 or 0 hours relative to inoculation.[19] The day 4 (plus 8 and 12) interval was selected as an interval at which spirochetes have begun to disseminate from the site of intradermal inoculation and arthritis is beginning.[3] The day 12 (plus 16, 20, 24, and 28) intervals were chosen to evaluate the effect of immune serum on established arthritis during the interval in which arthritis peaks (2 weeks) and begins to resolve (3-4 weeks) in immunocompetent mice.[3] Mice treated with immune serum prior to inoculation did not become infected, and mice treated with immune serum on days 4, 8, and 12 were cured of their infection and did not develop carditis or arthritis when examined on day 14. Mice treated with immune serum on days 12, 16, 20, 24, and 28 were infected, but arthritis had significantly resolved (Fig 17), unlike mice treated with normal mouse serum which had progressing arthritis at 30 days (Fig 18). To determine if immune serum would sustain the attenuated state of arthritis during persistent infection of *scid* mice, mice were also treated with immune serum on days 12, 18, 24, 30, 36, 42, 48, and 54, and then examined on day 60. Arthritis was nearly completely resolved in mice receiving immune serum, whereas mice treated with normal mouse serum had severe arthritis.[192]

Passive immunization studies in *scid* mice with immune serum from low dose infected mice revealed two important new phenomena: postinfection immune effects and arthritis modulating effects of immune serum. The post-infection immunity, in which *scid* mice could be cured of infection during the early stages of infection, contradicted the generally held belief that transfer of antibody after infection was ineffective. Russell Johnson established that hyperimmune serum against *B burgdorferi*, generated in rabbits, could be passively transferred to laboratory hamsters and protect them against challenge inoculation. He showed that such antiserum was protective only if given prior to or at the time of, but not 17 hours after, challenge inoculation.[193] Similar results were

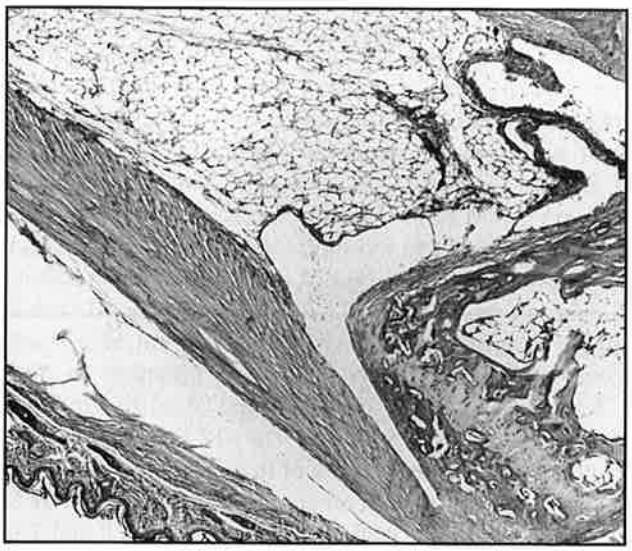

*Fig 17. Tibiotarsus of a C3H-*scid *mouse at 30 days after* B burgdorferi *infection. This mouse has been passively immunized with serum from an immunocompetent C3H mouse on days 12, 16, 20, 24, and 28 after infection. Arthritis has resolved, indicating that serum from infected immunocompetent mice contains antibody that can cause arthritis resolution.*

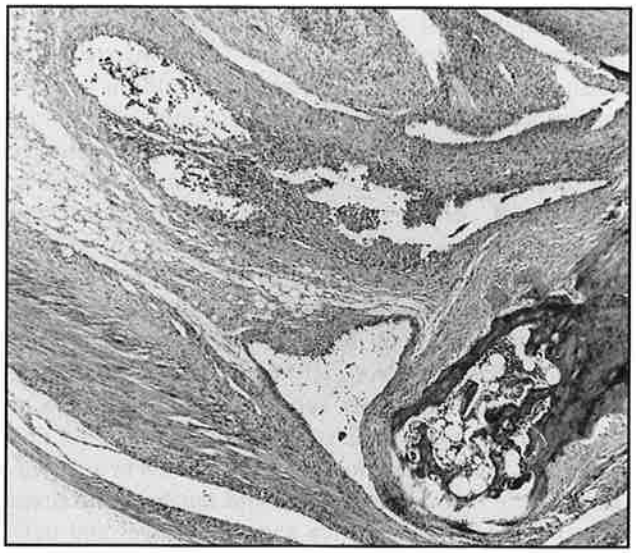

*Fig 18. Tibiotarsus of a C3H-*scid *mouse at 30 days after* B burgdorferi *infection. This mouse has been passively immunized with normal mouse serum at the same intervals of the mouse depicted in Fig 17. Note the severe arthritis in this mouse.*

demonstrated in *scid* mice, in which polyclonal hyperimmune serum to *B burgdorferi* or monoclonal antibody to OspA or B were protective when given before, but not after, challenge inoculation.[194,195]

The antibody profile in immune serum is important for interpreting these seemingly discordant findings. As discussed previously, immune serum from mice infected with low doses of spirochetes contains antibody directed against a limited repertoire of *B burgdorferi* antigens, which does not include OspA or Osp*B* Seroreactivity to at least 2 antigens, P39 and OspC, appears only in mice that become actively infected with spirochetes. Hyperimmune serum, generated in mice that have been hyperimmunized with nonviable *B burgdorferi*, contains strong reactivity to OspA and OspB, among many other antigens, but reacts poorly if at all to P39 or OspC. Since OspA and OspB antiserum has been shown to be passively protective in mice (discussed in the following), the artificial presence of OspA and OspB antibody in serum from high dose infected mice would obscure the effects of antibody to antigens of significance to the natural infection. Sera from mice infected with low doses of spirochetes, with host-adapted spirochetes delivered by tissue transplant or with tick-borne spirochetes all possess similar *B burgdorferi* immunoblot profiles.[18] Immune serum from low dose infected mice contains remarkably strong passive protective activity.[18-20,34] These observations suggest that infecting spirochetes elicit a different immune response than killed spirochetes. To test this assumption, immune serum from mice at 90 days and 15 months after low-dose infection was compared to the activity of hyperimmune serum generated against heat-killed *B burgdorferi* or hyperimmune serum against recombinant OspA, using the *scid* mouse protective, postinfection, and disease-modulating model in which mice were passively immunized with sera on day 0; days 4, 8 and 12; or days 8 and 12. The latter 2 antisera were selected to represent antibodies generated against *B burgdorferi* antigens expressed in culture. Treatment of *scid* mice with 90-day or 15-month immune serum had strong protective, postinfection and disease-modulating activity when given either before or after *B burgdorferi* infection; hyperimmune sera to heat-killed spirochetes or OspA was effective only if given on day 0 relative to inoculation, but not thereafter.[192] Thus, these results are concordant with other studies that have shown protective effects of hyperimmune serum to *B burgdorferi* and OspA prior to, but not after, inoculation, but underscore the more biologically relevant effects of immune serum from low-dose infected mice in post-infection and disease-modulating activity, which are likely to be important in both disease and persistent infection in the immunologically responsive host. A troubling observation was a study that tested the relative protective qualities of hyperimmune serum to *B burgdorferi* with immune serum from mice infected by tick-borne exposure. Both sera were shown to be passively protective in mice challenged by syringe with 10^6 spirochetes, but passive transfer of such sera (0.5 mL neat) protected mice against tick-borne challenge when administered no more than 1 day after tick attachment. If given 3 to 5 days after tick attachment, mice became infected.[196] Likewise, we have found that

passive immunization of mice with immune serum from low dose infected mice will protect mice against challenge inoculation with 10^7 syringe-borne spirochetes, but not against transplantation of tissue containing host-adapted spirochetes (unpublished observations). Tick-borne or host-adapted organisms appear to be refractory to protective and post-infection activities of immune serum, an issue that needs careful study.

Complement is probably not critical for these antibody-mediated events in the mouse. In vitro studies have suggested that complement is required for antibody-mediated *B burgdorferi* killing in hamsters,[197,198] but in vitro inhibition studies with immune mouse serum and monoclonal antibodies have indicated that spirochete killing can occur in the absence of complement.[123] No effect on infection or course of disease was found in low-dose *B burgdorferi*-infected, complement (C5)-deficient compared with complement-sufficient strains of mice, and passive immunization with immune serum was equally effective in complement deficient and sufficient mice.[199] These results indicate that antibody-mediated protection and disease resolution can occur in the absence of C5-dependent complement activation, but complement is likely to participate in antibody-mediated *B burgdorferi* killing.

Biologically relevant *B burgdorferi* antigens

This review has repeatedly emphasized that when mice are infected with initially antigenically subliminal doses of spirochetes, regardless of their source (culture, transplant, or tick), the ensuing antibody response is similar, as it reflects the response to organisms that replicate and disseminate in the host. Certain antigens, such as P39 and OspC, serve as markers of active infection, but it is unclear which antigens elicit biologically relevant antibody responses that are responsible for protective, postinfection, or disease-modulating activity. Although recombinant OspC has been shown to induce protective immunity in gerbils against *B afzelii* PKo[200] and in mice against *B burgdorferi sensu stricto* Son-188,[201] we cannot elicit passive or active immunity with recombinant OspC against *B burgdorferi sensu stricto* N40 (unpublished observations), the strain we use to infect mice to generate low-dose immune serum that is strongly protective. Furthermore, we have been unable to elicit protective immunity in mice with recombinant P39 (unpublished observations) or flagellin.[202] In spite of this, immune serum from low-dose infected mice at 2 weeks of infection has immunoblot reactivity almost exclusively to flagellin, P39, and OspC,[4,18,19] yet 2 week immune serum from mice infected with as few as 10 spirochetes is passively protective.[19]

A number of recent observations suggest that *B burgdorferi* undergoes significant changes within the mammalian host and there appear to be a number of novel antigens expressed exclusively in vivo that may be responsible for eliciting biologically relevant antibody responses. If this proves to be true, then immunoblots using cultured spirochetes would contain only antigens expressed in vitro and would thus fail to detect antigens expressed exclusively in vivo. P39 and OspC, which elicit detectable antibody responses only in actively infected mice, appear to represent antigens that are expressed in vivo and to a limited degree in vitro, and therefore can be seen on immunoblots, but other antigens may be totally cryptic in vitro. In support of this hypothesis, low-dose immune serum was found to label spirochetes in arthritic joint tissue from *scid* mice by indirect immunohistochemistry (Fig 19), but hyperimmune serum to heat-killed *B burgdorferi* or recombinant OspA (representing antigens expressed in vitro) did not label spirochetes.[18,192] In addition, hyperimmune serum to recombinant P39 and OspC also failed to label spirochetes in tissue sections (unpublished observations). Hyperimmune serum to viable *B burgdorferi* has also been shown to label spirochetes in tissues from humans and mice.[72,84] In addition, spirochetes have been labeled in tissue sections with antiserum to flagellin, an antigen expected to be present regardless of adaptive state.[76] A number of different *B burgdorferi* proteins have now been shown to be expressed exclusively in vivo, including 18 kDa EppA,[203] a 21 kDa protein,[154] a 22 kDa protein,[156] and an OspF homologue, K2.10.[204] By screening a *B burgdorferi* genomic expression library[154] with low-dose infected mouse serum, we have identified a number of other in vivo expressed proteins, but so far none have proven to be protective or disease modulating (unpublished observations). Identification of these biologically relevant antigens may prove to be a means toward eventual therapeutic immunization for treatment of Lyme disease and possibly more sensitive and specific serodiagnostic antigens.

Vaccine immunity and its relevance to biologically relevant in vivo antigens

A number of *B burgdorferi* proteins have been shown to elicit protective immunity in passively or actively immunized mice. OspA monoclonal antibodies passively protected *scid* mice when administered at the time of challenge inoculation,[194] and hyperimmunization of immunocompetent mice with native and recombinant OspA were shown to be protective against syringe challenge.[195,205,206] Passive immunization of mice with polyclonal or monoclonal antibodies to OspA have also been shown to be protective in *scid* and immunocompetent mice.[194,195,201,202,205] OspA immunization affords long-term protection against syringe challenge up to at least 150

days after immunization,[207] and is effective against tick-borne exposure.[25,208] Such studies have revealed that OspA immunization provides protection against a number of different *B burgdorferi* isolates, but not against isolates with OspA sequences that vary from the immunizing strain.[47,209] The degree of cross-protection appeared to be greater when immunized mice were challenged by tick-borne inoculation.[49,210] Active or passive immunization against flagellin elicited no protection,[194,202,211] whereas OspB immunization was protective, but not to the degree afforded by OspA vaccination.[129,202,212] OspC was shown to be protective against *B burgdorferi* isolate Son188 in mice immunized with OspC-Son188,[201] but we cannot elicit protective immunity in mice against the N40 strain, using OspC-N40 recombinant protein (unpublished observations). Other *B burgdorferi* antigens have been shown to have weak or no apparent protective activity, including OspD,[201] OspE, OspF,[48] a 21 kDa protein,[154] two 22 kDa proteins,[156,213] a 55 kDa protein,[214] and an 83 kDa protein.[201]

Although OspA is a good vaccine candidate and can elicit a strong protective response in appropriately immunized mice, low-dose syringe, transplant-borne, or tick-borne infection does not elicit a detectable OspA antibody response.[18,20,32] OspA is abundantly expressed on spirochetes in culture and within the midgut of ticks.[23,25] This would suggest that OspA is not expressed to a significant degree in the early stages of infection and thus host-adapted spirochetes should be refractory to OspA-induced immunity. To test this hypothesis, mice were actively or passively immunized against OspA, then challenged by syringe with cultured spirochetes or by transplantation of ear tissue from infected mice, containing host-adapted spirochetes. Mice challenged by syringe were protected, whereas mice challenged with host-adapted spirochetes were fully susceptible to infection and disease, indicating that spirochetes were completely unfettered by OspA immunity.[18] Recent experiments have shown that the loss of OspA by spirochetes occurs within the tick, prior to invasion of the mammalian host. OspA was demonstrable on spirochetes within the midgut of ticks,[23,25,215] but within hours of beginning a blood meal, spirochetes rapidly lost OspA and migrated to salivary glands, where OspA was no longer expressed on the organism.[26] When ticks fed upon OspA immunized mice, spirochetes were killed directly within the midgut of the tick, preventing infection,[25] but when OspA antiserum was passively administered to mice, it was protective prior to tick attachment and up to 36 hours after attachment, but not thereafter, indicating that spirochetes were no longer vulnerable to OspA antibody.[26]

These studies suggest that OspA may not be expressed in vivo; there is the fact that patients and mice seroconvert to OspA late in infection[4,57,136-142] and DR4+ patients with

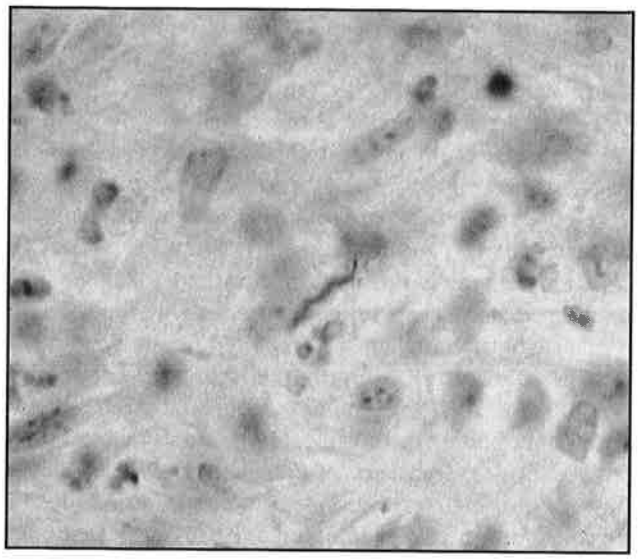

Fig 19. B burgdorferi *in synovial tissue of a* scid *mouse, labeled by indirect immunoperoxidase histochemistry with immune serum from an actively infected immunocompetent C3H mouse. Spirochetes do not label with hyperimmune serum generated in mice immunized with heat-killed* B burgdorferi *or recombinant OspC, OspA, or P39, suggesting that serum from actively infected mice recognizes antigens that may be expressed in vivo.*

antibiotic-resistant arthritis may develop an unusually strong humoral response to OspA and B, suggesting a possible role of MHC-restricted reactivity to OspA/B in the pathogenesis of chronic arthritis.[57] The possible association of immune reactivity to OspA and chronic disease has been explored in mice both hyperimmunized against OspA and in mice that are immunologically tolerant to OspA. Immunization of C3H mice with recombinant OspA at intervals after infection with *B burgdorferi* had no significant effect upon infection or disease, although immunization at an interval in which disease was resolving appeared to accelerate the resolution phase of disease[216]; this may be due to the nonspecific B cell mitogen and adjuvant effects of OspA.[53,173-176] Conversely, infection and disease were not altered in mice made immunologically tolerant to OspA or OspB. This was accomplished by creating C3H (disease susceptible) and C57BL (disease resistant) transgenic mice that expressed OspA or OspB under control of the MHC class I promoter. These mice did not elicit an immune response to OspA or OspB, respectively, when immunized with these proteins, but were fully immunoresponsive to other *B burgdorferi* antigens. Infection and disease were similar in transgenic and nontransgenic mice.[217]

The above studies with OspA show that *B burgdorferi* spirochetes undergo significant changes in protein expression within the tick and the infected host that have profound effects upon host immunity to the spirochete. There

is evidence for other major protein shifts, including low levels of OspC expression by spirochetes in the midguts of resting ticks and up-regulated expression of OspC in feeding ticks.[27,215] OspC is not abundantly expressed in culture, but spirochetes obtained from peritoneal lavage of actively infected mice appear to have up-regulation of OspC, based upon immunocytochemistry and RT-PCR for mRNA, during the first weeks of infection.[218] This correlates with the early, strong OspC antibody response of mice infected with low doses of spirochetes or by tick-borne infection.[18] Much needs to be learned about this very dynamic organism, with its significant changes in protein expression in culture, tick, and host. Such studies require careful consideration of the vector-spirochete-host interaction, with careful control of experimental variables. The mouse model affords the ideal conditions for such studies.

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Borrelia burgdorferi Infection of Inbred Strains of Mice Provides Insights Into Cellular and Molecular Parameters of Pathogenesis and Protection of Lyme Disease: A Viewpoint

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Key words: *Borrelia burgdorferi*, Lyme disease, mice, pathogenesis

SUMMARY

Borrelia burgdorferi sensu lato infection in the inbred mouse provides a valuable model of Lyme disease in that different forms of disease symptoms are induced according to the host strain. The initial finding that young or immunocompromised mice—but not their normal adult counterparts—develop clinical arthritis upon spirochetal challenge indicated that the development of disease is controlled by the immune system. However, when a number of mouse strains with severe combined immunodeficiency *(scid)* syndromes were analyzed, two groups with distinct disease patterns emerged: one group (CB-17.*scid*, C3H.SCID, NIH-3) being highly susceptible and developing most severe forms of chronic progressive arthritis; the other group (RAG-1D/D, RAG-2D/D) being much less susceptible, and showing, if at all, only mild forms of intermittent arthritides. The fact that the members of the two groups differ in their genetic background indicates the involvement of a gene(s) that regulates innate susceptibility or resistance.

By comparing highly susceptible *scid* mice with their coisogenic immunocompetent counterparts, again two groups with distinct disease patterns emerged: CB-17 mice (immunocompetent, resistant) do not show any signs of arthritis in response to spirochetes. In studies of passive protection using CB-17.*scid* mice, it was found that antibodies to outer surface lipoproteins of *B burgdorferi* are essential for the control of spirochetal infection and that T cells are critically involved in the generation of optimal protective antibody responses but not in the direct elimination of spirochetes from the host.

In contrast to CB-17 mice, C3H mice (immunocompetent, susceptible) are unable to control disease, despite their ability to generate protective antibodies with kinetics and quantities similar to those seen in resistant mice. A clue as to the underlying mechanism(s) was obtained by showing that the major histocompatibility (H-2) complex, particularly genes encoding class I molecules, contribute to the development of disease and that the H-2-determined susceptibility is more pronounced in strains of low susceptibility rather than in those of high susceptibility.

The conclusion—that mice with particular class I MHC genes develop T cells with pathogenic potential in response to spirochetes—was substantiated by the finding that elimination of $CD8^+$ but not $CD4^+$ T cells from susceptible mice leads to resolution of the arthritic symptoms. The recent finding that lipoproteins of *B burgdorferi* provide co-stimulatory signals to T cells, indicates that T cells of any specificity, beside those to spirochetal antigens, can contribute to inflammatory processes. Together, these results demonstrate that genetic traits for susceptibility or resistance of mice to spirochete-induced disease are dictated by both innate and T-cell genetic components. It remains to be determined, however, which cell population(s) governs the preferential activation of exacerbative or protective processes.

One of the most relevant findings of *B burgdorferi* infection in inbred mouse strains is the fact that all experimentally or naturally challenged recipients remain lifelong carriers of spirochetes, independent of their genetic background, their ability to control disease, or their potential to produce bactericidal antibodies. Studies employing passive immunization protocols have shown that eradication of spirochetes from infected mice or infesting ticks only occurs when protective antibodies were given before or together with, but not after, the challenge. These results clearly indicate that sterile immunity against Lyme disease is only achieved by prophylactic

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immunization strategies. A vaccine formula on the basis of the recombinant outer surface lipoprotein A, OspA, is being tested in phase III clinical trials for safety and efficacy to mediate full protection against Lyme disease.

CRITICAL APPRAISAL OF STUDIES

Pathogenesis and protection in inbred mice infected with *B burgdorferi*

Lyme disease is caused by spirochetes of the *Borrelia burgdorferi sensu lato* complex, including various species such as *B burgdorferi sensu stricto, Borrelia garinii,* and *Borrelia afzelii*, which are transmitted to humans by infected ticks.[1-3] The pathobiology of the infection is fraught with a number of serious problems, such as limitation of diagnostic tools, unpredictable onset and manifestation of disease symptoms, insufficient immunological control, heterogeneity of spirochetes and their potential to persist in the host, as well as unreliability of therapy.[1,4] Only the disclosure of the genetic and structural basis of these aspects, both with respect to the spirochete and the mammalian host, will allow us to design optimal prophylactic and therapeutic regimens for an efficient control of disease and infection.

Mice are the main reservoir hosts for a number of genospecies of the *B burgdorferi sensu lato* complex, comprising those that are either pathogenic (ie, *B burgdorferi sensu stricto, B garinii,* and *B afzelii*) or nonpathogenic (*Borrelia japonica*) for humans[5,6] and are critical in the enzootic cycle of the spirochetal infection.[7] The principal hosts, such as the white-footed mouse *Peromyscus leucopus* (northeastern United States), the European mouse species *Apodemus flavicollis* and *Apodemus sylvaticus* and the vole species *Clethrionymus glareolus* become persistently infected following natural or experimental inoculation but do not develop overt clinical symptoms.[7-10] The apparent reservoir competence of these small rodents for the various species of the *B burgdorferi sensu lato* complex is startling in view of their immunoreactivity and, in particular, their capacity to develop vigorous antibody responses to a wide spectrum of *Borrelia* antigens.[11] Obviously, *B burgdorferi* has developed suitable strategies that ensure its survival in natural mammalian hosts and its transmission between reservoir hosts.

To investigate the genetic basis of cellular and molecular events in *Borrelia*-host interaction, inbred strains of mice have been used that are genetically homogeneous and therefore sufficiently uniform in their characteristics of infection profiles.[12-16] In the following, the results from independent laboratories are summarized and the relevance to the emerging principles for human Lyme disease will be discussed. It should be stressed that such a comparison is somewhat difficult because variables of *B burgdorferi* strains and passage history, mouse strains, mouse age, spirochetal dose, and route of inoculation all influence the results to a remarkable extent. The point concerning the different age of mice used (3 days to 10 weeks) and their apparently distinct immune competence is of particular importance in view of the potential contribution of innate and adaptive defense mechanism(s) to the development or the control of disease (see below). Moreover, even when the same inbred mouse strains from different suppliers are analyzed, variations may occur because of small differences in the genetic background and/or in the health status within individual colonies.[17]

Early studies have shown that adult animals of various inbred strains of mice develop, if at all, only weak clinical signs of arthritis after experimental infection with *B burgdorferi*. On the other hand, young mice are more susceptible to the development of arthritis.[18-20] Together with previous results on the increased susceptibility of neonatal rats[21] and irradiated hamsters[22] to *Borrelia*-induced arthritis, the data indicated that in rodents, disease mainly develops in the absence but not in the presence of an appropriate immune response. This conclusion was further substantiated when it was shown that, upon inoculation with *B burgdorferi, scid* mice of either BALB/c.*Igh*^{b} (C.B-17[19,23]) or C3H background[24] or the immunodeficient NIH-3 mice (lacking NK cells in addition to B and T cells[25] develop severe persistent unremitting forms of arthritis, myositis, and carditis (Table 1). The chronic progressive inflammation of organs is associated with the infiltration of mononuclear cells and the presence of spirochetes in the affected tissues.[19,23-25] It became clear that only viable but not killed spirochetes are pathogenic in mice and that the severity of organ destruction is related to the number of microorganisms in the affected tissues.[14,19,26] These findings are noteworthy for two reasons: first, they imply a pivotal role of the immune system in the control of spirochete-induced pathology in mice; and second, they emphasize that the inflammatory processes initiated and propagated during *B burgdorferi* infection in various organs, such as joints, heart, and liver may develop in the absence of B and T cells.[19,23,27]

However, when two *scid*-type mouse mutant strains of the C57BL/6 genetic background, which lack either the V(D)J recombination activation genes RAG-1 (RAG-1D/D)[28] or RAG-2 (RAG-2D/D)[29] and consequently do not have any mature B and T lymphocytes, were infected with *B burgdorferi* they developed, if at all, only mild forms of intermittent arthritides (Table 1). The fact that the low-susceptible (RAG-1D/D and RAG-2D/D) and high-susceptible (C3H, BALB/c.*Igh*^{b}, NIH) *scid* strains differ in their genetic background, clearly indicate that resistance against and susceptibility for spirochete-induced disease

Table *1*

Development of Arthritis in Mouse Strains

Mouse Strain	H-2 haplotype	Background Genes	Arthritis	*B.b.* re-isolation	Antibody Formation
C3H. *scid**	k	C3H	+++++	++	-
C.B-17. *scid**†	d	BALB/c.*Igh*^{b}	++++	++	-
NIH-3‡	-	NIH	+++	++	-
RAG-1 D/D†	b	C57BL/6	Ý	n.d.	-
RAG-2 D/D†	b	C57BL/6	Ý	+	-
AKR/N†	k	AKR	+++	+	+
C3H/HeJ(HeN)*†	k	C3H	++	+	+
C.B-17*†	d	BALB/c	-	+	+
BALB/c†	d	BALB/c	-	+	+
BALB/cByJ*	d	BALB/c	Ý	+	+
DBA/2†	d	DBA/2	-	+	+
CAL.20†	d	BALB/c	-	+	+
B10.D2†	d	C57BL/10	-	+	+
NIH‡			-	n.d.	n.d.
C57BL/6*†	b	C57BL/6	Ý(+)*	+	+
RAG-1 D/N†	b	C57BL/6	Ý	n.d.	+
SWR/J*	q	SWR	+Ý	n.d.	+
SJL/J*	s	SJL	+	n.d.	+
C57BL/6†	bbbb	C57BL/6	Ý	+	+
B10†	bbbb	C57BL/10	Ý	+	+
B10.D2†	dddd	"	-	+	+
B10.BR†	kkkk	"	+	+	+
B10.AQR†	qkkd	"	Ý	+	+
B10.A(5R)†	bbkd	"	Ý	+	+
B10.A†	kkkd	"	+	+	+
C3H/HeN§	k	C3H	++	n.d.	n.d.
C3H.SW§	b	"	++	n.d.	n.d.
B10§	b	C57BL/10	Ý	n.d.	n.d.
B10.BR§	k	"	Ý	n.d.	n.d.

Combined data from:

**Barthold et al, 1990, 1991; †Schaible et al, 1989, 1991; Simon et al, unpublished; ‡Defosse et al, 1992; and §Yang et al, 1992.*

Age of mice at the time of infection:

3 days, 3 to 4 weeks (Barthold et al, 1990, 1991); 6 to 10 weeks (Schaible et al, 1989, 1991); 5 to 6 weeks (Yang et al, 1992).

RAG-1 D/D, RAG-2 D/D mice can be described as "non-leaky scid *mice" on the C57BL/6 background.[28,29]*

Isolates of B burgdorferi *used, numbers of spirochetes injected and route of infection: N40, 1x10^4 id (Barthold et al, 1990, 1991); ZS7, 1x10^8 sc (Schaible et al, 1989, 1991); N 40, 2x10^6 id (Yang et al, 1992); 297, CT-1, 1x10^7 im (Defosse et al, 1992).*

Ranking of clinical arthritis: the severity of symptoms of clinical arthritis in independent studies has been adjusted by the authors for sake of clarity: + to +++++ = increasing severity of arthritic symptoms; Ý = marginal arthritic symptoms; - = no arthritic symptoms.

Re-isolation of spirochetes: + = at least once during infection; antibody formation detected by IFA, ELISA, and/or Western blot analysis.

is regulated by a gene(s) determining innate immunity. This is reminiscent of the control of susceptibility and resistance in mice infected with intracellular parasites, such as *Mycobacterium bovis*, *Salmonella thyphimurium,* and *Leishmania donovani* by two alleles of a single gene, termed *Nramp1*.[30] *Nramp1* encodes an integral membrane protein of macrophages and affects their capacity to restrict intracellular replication of the parasites. It is possible that host susceptibility/resistance to infection with the extracellular agent, *B burgdorferi,* is also controlled by differential bactericidal activities of macrophage populations. In fact, it was shown that macrophages are able to phagocytose and readily kill *B burgdorferi* organisms in vitro.[31-33] The question whether the killing process is mediated by oxygen intermediates, nitric oxid, proteases, and/or other yet unknown effector molecules is still debated.[33-36] However, the finding that susceptibility and resistance of mice to *B burgdorferi* infection do not match the above mentioned *Nramp1* alleles suggests a distinct genetic basis and mechanism for the control of spirochete-

induced disease in mice.

The early conclusion that the immune system is critical in preventing development of disease during *B burgdorferi* infection was further challenged when the various *scid* mouse strains were compared with their coisogenic immunocompetent counterparts (Table 1). Whereas infected C.B-17 mice did not show any signs of clinical arthritis or pathological changes in joints or other organs,[18] C3H (HeJ/HeN) mice developed arthritis, which was most severe during the first weeks, progressed over a period of several months and then declined.[24,37] In subsequent experiments in which cellular and humoral elements of the C.B-17 immune system were transferred to C.B-17. *scid* mice, it became clear that bactericidal antibodies to the outer surface lipoprotein A (OspA) of *B burgdorferi*, and to a lesser extent also to OspB, are main effector molecules for the control of disease and that T cells are critical for the generation of optimal humoral responses but not for the direct elimination of spirochetes from the host.[12,38-41] The demonstration that protection against disease also can be transferred passively to immunocompetent recipients by presensitized T cell populations[42] supports this contention. Studies on active immunization with preparations of native or recombinant Osp showed that besides OspA and OspB,[43-45] OspC also provided protection in immunocompetent mice[46] and gerbils.[47]

The inability of C3H/HeJ and C3H/HeN mice and mice with identical H-2^k haplotype (AKR/N) but different genetic background (AKR) to control the development of arthritis (Table 1) was not due to an inappropriate immune response. All three strains generated humoral immune responses during infection, with kinetics, titers, and quality similar to those seen in resistant C.B-17 mice and with similar capacity to control spirochetes upon transfer into *scid* mice[14,37] (Schaible and Simon, unpublished data, 1995). These data indicate that the high level of innate susceptibility of these mouse strains to disease might override the protective potential of acquired immunity or that the immune response itself contributes to the pathogenic process(es) or both.

To get more information on the interrelation between innate and immunogenetic traits (H-2 haplotype, *Igh* allotype) in the control of *B burgdorferi*-induced arthritis, additional mouse strains, including H-2 congenic inbred strains, were analyzed (Table 1). It was found that independent of their innate susceptibility/resistance, all infected mice produced comparable patterns of antibodies, with respect to kinetics, specificities, and protective potential, as revealed by passive transfer experiments[14,37] (Schaible, M.M.S., unpublished data, 1995). However, all H-2^k mice with genetic backgrounds of either strain AKR, C3H, or C57BL/10 developed chronic progressive arthritis, although to various degrees, whereas all H-2^d mice on the backgrounds of either BALB/c, DBA/2, or C57BL/10, did not display overt clinical symptoms.[37] In an independent study, a more severe form of clinical arthritis was observed in C3H/HeJ mice[24] and BALB/cByJ mice were found to be susceptible to disease.[24] The discrepancy between the two studies may be due to the fact that in the latter, mice were not fully immunocompetent because of their young age (between 3 days and 3 weeks).[24] In addition, C57BL/10 mice of the H-2 haplotypes b,j,r,s[37] or SWR/J and SJL/J[20] mice developed arthritides of mild to intermediate intensity and variable duration. There was no obvious influence of the Ig allotype on the clinical course of infection in the mouse strains tested.[37]

These results clearly indicate an influence of the H-2 complex on pathogenesis and protection of *B burgdorferi* infection. However, the contribution of this genetic trait seems to depend on the level of innate susceptibility. This is supported by the finding that two congenic strains of the high-susceptible C3H background with the H-2 haplotypes k and b developed arthritis of the same severity,[17] whereas the two corresponding H-2 congenic strains of the low-susceptible C75BL/10 background had a distinct disease pattern: those with the H-2^k haplotype developed chronic arthritis and those with the H-2^b haplotype, if at all, only mild forms of arthritis.[37] However, in an independent study it was found, that C57BL/10 mice of both haplotypes, k and b, developed only moderate arthritis.[17] Although the reason for the latter discrepancy is unknown, it may be due to the use of mouse strains from different suppliers and/or of distinct *B burgdorferi* isolates.

More detailed studies on the role of H-2 genes in the development of arthritis using H-2 recombinant strains of C57BL/10 background indicated that the disease is associated with MHC class I rather than MHC class II genes,[37] thus involving $CD8^+$ T cells. This conclusion is supported by experiments, in which susceptible C3H/HeJ mice were depleted of either of the two T cell subsets, by treatment with anti-CD4 or anti-CD8 antibodies prior to infection. It was found that the severity of arthritis was ameliorated in mice lacking $CD8^+$ but not in those lacking $CD4^+$ T cells.[48]

We would thus like to propose the following sequence of events regarding innate and immune-related responses in the pathogenesis and protection of *B burgdorferi* infection in mice: early during infection and mainly independent of the host's genetic background, spirochetes disseminate from the site of the tick bite and directly induce inflammatory lesions in distant organs by breaching the vascular endothelium and invading the underlying tissues. This scenario is supported by several findings: 1) spirochetes are able to bind host derived enzymes (plasminogen/ plasmin) in vitro.[49-52] The surface-bound proteolytic activity, which is not inhibited by serum-derived inhibitors, facili-

tates their potential to traverse endothelial cell layers and to degrade basal membrane constituents[49-52]; 2) attachment of spirochetes to the vasculature in affected tissues leads to early activation of endothelial cells, as characterized by the upregulation of adhesion molecules, such as E-selectin, P-selectin, ICAM-1, and VCAM-1,[53,54] resulting in the attachment and extravasation of blood-borne leukocytes[54,55]; 3) spirochetal infection also leads to neo-vascularization in affected synovial tissues of *scid* mice[53]; 4) interaction of spirochetes with tissue-derived cells leads to the production of pro-inflammatory cytokines, such as IL-1β, TNFα, and IL-6[56]; and 5) whole spirochetes as well as enriched preparations of their lipoproteins and glycolipids directly induce macrophages to produce IL-1β, TNF-α, and IL-6 in vitro.[57-59] (Honarvar, Modolell, and Simon, unpublished data, 1995). Thus, one could envisage that in the early phase of infection different patterns of cytokines are produced by first-line defense cells such as fibroblasts, macrophages, endothelial cells, and natural killer cells in genetically distinct mice and that it is this initially generated milieu of cytokines that determines the further course of pathogenesis and infection. In fact, it was shown before in other disease models that the cytokine milieu generated in the early phase of infection may be crucial in determining susceptibility or resistance to disease.[60,61] It is also possible that the initially induced milieu of cytokines contributes to the differential development of T cell subsets with distinct pathogenic potential.[60] In this respect, it was interesting to learn that presensitized lymph node cells from disease-susceptible C3H mice were found to produce high levels of IFN-γ and low amounts of IL-4, when restimulated in vitro, whereas those of disease-resistant BALB/c mice produced only marginal or low amounts of IFN-γ and IL-4.[62,63] The suggestion that the two cytokines have opposing effects on the development of arthritis is supported by in vivo studies, showing that treatment of mice with neutralizing anti-IFN-γ antibodies reduced both joint swelling and spirochete burden whereas similar treatment with anti-IL-4 antibodies led to increased joint swelling and higher spirochete burden than in control mice.[62,63]

How, then, could IFN-γ induce organ pathology such as arthritis, carditis, and hepatitis in susceptible AKR/N and C3H mice? IFN-γ is known as a potent proinflammatory mediator.[64] It induces the release of a battery of monokines, such as IL-1, TNFα, and IL-8, from macrophages which have a profound effect on subsequent events by activating vascular endothelium and by increasing the influx of leukocytes into inflammatory foci.[27,64] This milieu would not only facilitate extravasation of neutrophils but also of macrophages, plasma cells, and activated and/or memory but not naive T cells. In fact, T cells have been observed in tissue sections of infected AKR/N mice.[14,27] Moreover, it was found that *B burgdorferi* organisms, which are present in actively inflamed tissues,[14,23,65] directly stimulate, via their lipoproteins and glycolipids, macrophages and B cells, to produce bioreactive molecules or antibodies, respectively,[34,35,66-68] and provide co-stimulatory signals for T cells both directly or indirectly via antigen-presenting cells.[69] Together, these findings suggest that the pathological processes are maintained by direct interaction of spirochetes with tissue-associated and/or infiltrating cells. Since pre-activated T cells infiltrate inflammatory foci, irrespective of their antigen-specificity,[70] it is expected that T cells specific for spirochetal, third party, and most probably also to auto-antigens can expand in infected tissues and contribute to pathology.[64] IFN-γ may also play a direct role by elevating the cell surface expression of MHC class I antigen and thus induce $CD8^+$ T cell-mediated pathology as a consequence of breakdown of self-tolerance.[71]

The most startling finding of *B burgdorferi* infection in inbred strains of mice is that spirochetes persist in all recipients, irrespective of their immunocompetence and their potential to control disease.[10,13-16,72] In this respect, the experimental infection of inbred strains of mice—both by needle injection or tick infestation—reflects the situation in natural hosts and, most probably, also in patients with untreated Lyme disease. It became clear that spirochetes are able to evade elimination, even in the presence of highly potent borreliacidal antibodies.[10,13-16,72,73]

Obviously, a rational strategy to avoid persistent infection is the eradication of spirochetes at the time of inoculation, ie, during the blood meal of the infected tick. Previous findings that hamsters can be protected against *B burgdorferi* by active and passive immunization clearly indicated that immunoprophylaxis against Lyme disease is possible.[74,75] Recently, a vaccine consisting of freeze-dried whole *Borrelia* organisms (Bacterin) has been developed to protect animals against infection.[76] However, the suitability of such a complex vaccine formula for human use is problematic, due to possible side effects by endogenous, ill-defined compounds. We and others have shown that resistance to experimental and tick-mediated infection with *B burgdorferi* can be passively transferred to mice by antibodies to individual outer surface lipoproteins, such as OspA, OspB, and OspC, provided that the antibodies were applied in sufficient quantity either before or at the time of but not after infection.[13-16,77] In this respect it was intriguing to find that antibodies to OspA also eliminate spirochetes within the tick gut, when transferred during infestation.[78] Extensive studies revealed that among other structures with immunogenic and protective potential, preparations of native and recombinant lipidated OspA, are most promising for immunoprophylaxis[38,40,43,79,80] and that high levels of protective antibodies can be maintained in mice for months following active immunization with recombinant preparations of lipidated OspA.[81] Most noticeably, because of its

lipid portion, additional complex adjuvants seem to be dispensable for the establishment of a protective immune status in mammals, including humans.[82]

The immunogenicity of recombinant vaccine formulations, including lipidated OspA, has been approved in previous clinical studies,[83] and a formula based on lipidated OspA is presently being tested for its efficacy in phase III clinical trials. Despite these encouraging findings, however, the application of OspA as a vaccine still is dependent on an assessment of its safety and its ability to provide full protection against all members of the *B burgdorferi* species and to prevent escape mechanism(s) of the spirochetes. Previous studies have implicated nonspecific and specific humoral and cellular immune responses to lipidated OspA, including mitogenic activation of B cells[66-68] and co-stimulation of T cells,[69] in the pathogenesis of Lyme arthritis, both in mice and man.[84,85] Although it has still to be consolidated that the lipid portion of OspA contained in the vaccine formulation is without any risk, more recent data on the development of arthritis in mice tolerant to OspA, suggest that the protein moiety of OspA is not involved in *Borrelia*-induced pathogenesis.[86] The other critical aspect about the efficacy of OspA as a vaccine is concerned with the extensive heterogeneity of OspA proteins within the species of *B burgdorferi*.[87,88] Two studies, using OspA recombinant proteins, showed that antibody-mediated crossprotection against experimental challenge was not efficient between heterologous strains, despite extensive crossreactivity of the induced antibodies with heterologous OspA.[89,90] On the other hand, the finding that a monovalent OspA vaccine protects mice from tick-borne infection with heterogeneous *B burgdorferi* from different geographic regions, including those of the genospecies *B burgdorferi sensu stricto* and *B afzelii*,[91] remains controversial.[92] Other concerns relate to the obvious potential of spirochetes to alter their phenotype in response to environmental and/or host-derived factors.[93-96] A recent experiment demonstrated that OspA is highly expressed in tick-derived spirochetes but downregulated upon their transmission into mice.[93] Together, these findings emphasize the necessity to search for additional *B burgdorferi* molecules as targets for protective antibodies[97] that meet the requirements for a save and comprehensive vaccine against Lyme disease, either on their own or in combination with OspA.

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Transmission of *Borrelia burgdorferi sensu lato* by Reservoir Hosts

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Key words: *Borrelia burgdorferi sensu lato*, reservoir hosts, transmission

INTRODUCTION

Important information on the transmission of *Borrelia burgdorferi sensu lato* has recently been obtained from studies using laboratory and natural reservoir hosts. This review paper focuses on natural reservoir host species as animal models used in laboratory studies of transmission. Relevant information on immune processes acting both at the host-pathogen interface and at the tick-host interface are presented and discussed in relation to spirochete transmission.

The taxon *B burgdorferi sensu lato* comprises at least eight genomic groups of spirochetes, most of which seem to cause Lyme borreliosis in humans. These groups are commonly referred to as genospecies.[1,2] Considerable heterogeneity has been described for strains even within species.[3-5] Most of the strains isolated in North America belong to the genospecies *B burgdorferi ss,* DN127 and 21123. For central and western Europe, the genospecies *B burgdorferi ss, Borrelia garinii,* and *Borrelia afzelii,* as well as a group designated VS116, have been shown to be prevalent in tick and vertebrate host populations.[1-3,6-8] Increasing evidence suggests the existence of further distinct genotypes within the *B burgdorferi sl* complex in the United Kingdom.[9] Phylogenetic analysis of isolates suggests that horizontal gene transfer of genetic material between strains is unlikely to occur and that *B burgdorferi sl* is clonal.[4]

A large number of vertebrate species is involved in the transmission cycles of *B burgdorferi sl.*[10-18] Transmission coefficients of *B burgdorferi sl* between ticks and tick hosts are important parameters in the transmission cycle(s) that determine the transmission dynamics of these tick-borne pathogens in nature.[19] According to the particular tick-host system and the experimental or the ecological conditions, the intensity of spirochete transmission may vary substantially.

Many studies on spirochete transmission have been conducted within the framework of vaccine development using mice with defined genetic background or other animals such as hamsters, gerbils, dogs, or rhesus monkeys.[20-30] This paper reviews recent research on spirochete transmission using laboratory and natural reservoir hosts.

TRANSMISSION OF *B BURGDORFERI SL*

Most of the tick species known to be vector-competent for *B burgdorferi sl* belong to the *Ixodes ricinus*-species complex (subgenus *Ixodes). Ixodes uriae* (subgenus *Ceratixodes)* and *Ixodes hexagonus* (subgenus *Pholeoixodes)* are important exceptions.[31,32] Infected ticks may transmit *B burgdorferi sl* and establish an infection in a host. The probabilities with which an infected tick establishes an infection in a vertebrate and an infected host passes spirochetes to feeding ticks are referred to as transmission coefficients.[19] These values may, theoretically, range from 0 to 1.0. The host-to-vector transmission coefficient is measured most commonly as the percentage of ticks acquiring a *B burgdorferi* infection from a host.

The information currently available suggests that coefficients of transmission of *B burgdorferi ss* to *Peromyscus leucopus* (white-footed mouse) by *Ixodes scapularis* nymphs may be close to 1.0.[33] Preliminary information indicate that the transmission coefficients for *B afzelii* transmitted to gerbils by *I ricinus* nymphs is

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Table
Important Natural Reservoir Hosts Used as Animal Models of Lyme Borreliosis Transmission

Apodemus flavicollis (yellow-necked mouse)
Apodemus sylvaticus (wood mouse)
Clethrionomys glareolus (bank vole)
Peromyscus leucopus (white-footed mouse)
Sciurus carolinensis (grey squirrel)
Phasianus colchicus (pheasant)

These mammalian and avian species are examples of species that have been used as animal models to study Borrelia burgdorferi sl *transmission, both in the laboratory and in the field. Most of our knowledge on immunity as a factor in spirochete transmission has been obtained from rodents.*

similarly high (O.Kahl, PhD, unpublished data, 1995) It has been recorded that transovarially infected larvae of *Ixodes hexagonus* may transmit spirochetes to laboratory mice.[32] At present, there is no direct evidence on whether infected larvae of *Ixodes scapularis*[34] or of *I ricinus* may establish systemic infections in natural hosts. There is, however, theoretical and epidemiological evidence suggesting that transovarially infected *I ricinus* larvae are much more important in the transmission cycle(s) of *B burgdorferi sl* in Europe than previously thought.[10,18,19,35,36]

Many vertebrate species of America and Europe have been investigated for their capacity to serve as reservoirs to *B burgdorferi sl* both in the laboratory and in the field. High host-to-vector transmission coefficients have been documented for some rodent species, eg, the American species *P leucopus*[37] and the European species *Apodemus flavicollis* (yellow-necked mouse), *Apodemus sylvaticus* (wood mouse) and *Clethrionomys glareolus* (bank vole).[13,16,18,38-40] It appears that infected *P leucopus* and *C glareolus* infect ticks more efficiently than do mice of the genus *Apodemus*.[11,18,33,37,38,40,41]

In some parts of the range of *I ricinus,* other vertebrates, such as grey squirrels *(Sciurus carolinensis),* birds (eg, species of the families *Turdidae* and *Gallinaceae*), or hares *(Lepus timidus),* are important as hosts for immature ticks. Epidemiologic data show that these groups of tick hosts are susceptible to *B burgdorferi sl* and that they contribute to spirochete infection in ticks.[14,15,17,31,42-44] We recently established an avian model of *B burgdorferi sl* transmission in the laboratory using pheasants *(Phasianus colchicus).* This avian host was found to be susceptible to different genospecies of *B burgdorferi sl* and to transmit the agent to *I ricinus* nymphs with transmission coefficients ranging between 0.1 and 0.6 for more that 10 weeks postinfection (K.K., unpublished data, 1995).

For America and Europe, deer are considered to be incompetent as reservoir hosts.[16,45] Experimentally inoculated white-tailed deer *(Odocoileus virginianus),* however, have been described to pass spirochetes to *I scapularis.*[46] A recent study from Japan indicates that Sika deer *(Cervus nippon yesoensis)* allow localized skin infections with *B burgdorferi sl* at tick-feeding sites.[47] This points to the possibility that ticks may occasionally acquire spirochetal infections from deer when cofeeding with infected ticks, which has been shown to occur in laboratory mice.[48] However, the relative contribution of this mode of horizontal transmission to the basic reproduction number (Ro) of *B burgdorferi sl* in nature has not been calculated so far.

No experimental information is available on the relative transmissibilities of the various genospecies of *B burgdorferi sl* between the known vector and reservoir species. Epizootiologic data from Europe suggest an association of *B garinii* and *B afzelii* with birds and rodents, respectively.[6,42]

As the available data on coefficients of transmission vary substantially with the tick-host system, with the particular *B burgdorferi sl* strain, and, unfortunately, with the research team, the following sections attempt to identify factors that influence spirochete transmission.

THE ROLE OF IMMUNITY TO *B BURGDORFERI SL* AND TRANSMISSION

Laboratory conditions

Immune responses to *B burgdorferi sl* have been analyzed mainly in rodents,[25,40,49-51] dogs,[30,52] humans,[5] and in rhesus monkeys.[27] In most experimental studies *B burgdorferi sensu stricto* was used. Various studies revealed a remarkable difference in the quality of the antibody response to *B burgdorferi sl* after infections induced by needle inoculation compared with that after infections established by tick bites.[22,23,27,53] In particular, antibodies to the outer surface proteins OspA and OspB are usually lacking after tick-borne infection, but generated after intradermal infection. The immune response to *B burgdorferi ss* has been analyzed in the most important natural rodent hosts of Lyme borreliosis in western Europe, ie, *C glareolus, A flavicollis,* and *A sylvaticus.*[40] Tick-borne infections with this European isolate of *B burgdorferi ss* did not induce antibodies to OspA, OspB, and OspC, whereas antibodies to these outer surface proteins were consistently generated in all three rodent species inoculated by needle with either $1x10^6$ viable or $1x10^8$ irradiated *B burgdorferi ss.*[40]

It has been suggested that the generation of antibodies to the outer surface proteins is associated with the route of infection[53] or confined to needle-infection with cultured spirochetes.[23] It has been demonstrated that the expression

of the outer surface proteins of *B burgdorferi sl,* in particular OspA, is not downregulated in ticks.[54,55] Another study indicated that the generation of antibodies to OspA and OspB in mice is dose-dependent as antibodies to OspA were detected in mice inoculated with >10 000 but not with <10 000 viable spirochetes.[51] A subsequent study, analyzing the immune response of mice naturally infected or infected with low numbers of *B burgdorferi ss,* showed that the sera of both groups (ie, infected by ticks or with low numbers of cultured spirochetes, respectively) contained antibodies to a novel (outer surface) protein of *B burgdorferi ss* designated pG.[51,56] Antibodies to this protein cannot be detected by conventional Western blot analysis using whole cell lysates as this protein is exclusively expressed in vivo.[56]

The similarity of the immune response of rodents infected with low numbers of spirochetes by needle and that seen after tick-borne infection may indicate that a tick delivers fewer than 10 000 spirochetes into the skin of a host spread over several days. However, it is also possible that tick saliva modulates the dose-dependent pattern of the immunoreactivity to antigens of *B burgdorferi sl,* a question that has not been addressed.

B burgdorferi infections may persist lifelong in laboratory and natural rodent hosts.[40,57] The level of OspA- and B-specific antibodies generated in the course of infections appears to influence the host-to-vector transmission; a study using laboratory mice has shown that the infectivity of animals infected by ticks was significantly higher than the infectivity of mice inoculated with high numbers of cultured spirochetes by needle, which was inversely correlated with the presence of antibodies to OspA and B.[53] Similar results have been obtained using *A flavicollis, A sylvaticus,* and *C glareolus.*[40] Irrespective of the particular route an infection is initiated in a host, the overall magnitude of the immune response to OspA or to whole spirochetes has been observed to vary with the animal strain or species.[40, 50] For natural rodent hosts from Europe analyzed so far, *C glareolus* is a lower humoral and cellular respondent to *B burgdorferi ss,* allowing higher transmission coefficients to xenodiagnostic ticks than members of the genus *Apodemus.*[40]

The presence of antibodies to OspA, OspB, and OspC in hosts also influences the vector-to-host transmission, a fact that has been exploited for vaccine development.[20,21,25,30,58,59] The mechanisms by which elements of the hosts' immune system influence or prevent spirochete transmission between ticks and hosts are not understood fully. Some antibodies to OspA have been shown to be borreliacidal, ie, they directly kill *B burgdorferi* in vitro.[60] Complement-independent as well as complement-dependent effector mechanisms may act within the vertebrate host.[20,27,61,62] However, elements of the host's immune system may also kill borreliae within feeding ticks. This was first shown for ticks feeding on laboratory mice immunized with outer surface proteins.[28,58,63] The same phenomenon has been observed in preinfected *I ricinus* immatures fed on a natural host species, *A flavicollis* (the yellow-necked mouse), immunized with recombinant lipidated OspA (K.K., unpublished data, 1995). The protection of these animals and elimination of spirochetes from feeding ticks has been associated with antibodies to an epitope defined by the monoclonal antibody LA-2 (unpublished observation). In contrast, no elimination of *B burgdorferi ss* from *I ricinus* was observed when the animals had been injected with $1x10^8$ whole irradiated spirochetes.[40]

The influence on the transmission of antibodies to outer surface proteins of *B burgdorferi sl* other than OspA and OspB, such as OspC, OspD, OspE, OspF, and pG, is less clear, but it has been found that some of these antigens confer partial or full protection to challenge infections with homologous strains in laboratory mice or gerbils.[21,25,56,63]

At present, no laboratory study has investigated the immune response in hosts infected with more than one genospecies of *B burgdorferi sl* and its effect on spirochete transmission. The question arises whether the various genospecies are preferentially transmitted to ticks by particular host species.

Natural conditions

Is there a role for antibodies to the outer surface proteins of *B burgdorferi* as a factor in determining spirochete transmission between ticks and hosts under natural conditions?

Recently, two studies have analyzed the immunoreactivity to *B burgdorferi sl* antigens of sera from wild rodents. A study from Europe has shown that 8% of the sera of seropositive rodents trapped in a particular "hot spot" of Lyme borreliosis in Germany recognized proteins of *B burgdorferi ss* of 31 kDa (Fig), whereas no antibodies to proteins within this molecular mass range were detected in seropositive sera of animals from low endemic sites.[18,40] Due to the high infection prevalence of ticks in this highly endemic biotope, a fraction of the rodent population is exposed to repeated bites of *Borrelia*-infected ticks during the animals' life span. The presence of antibodies to protein(s) of 31 kDa may be caused by repeated immune stimulation following superinfections with borreliae.[18,40] These data indicate that the intensity of spirochete transmission in endemic sites (ie, the "entomological inoculation rate") influences the pattern of immunoreactivity against *B burgdorferi sl* antigens. In addition, a positive correlation between the number of questing, infected ticks and the seroprevalence was found for *A flavicollis* and *A sylvaticus,* but not for *C glareolus.*[18]

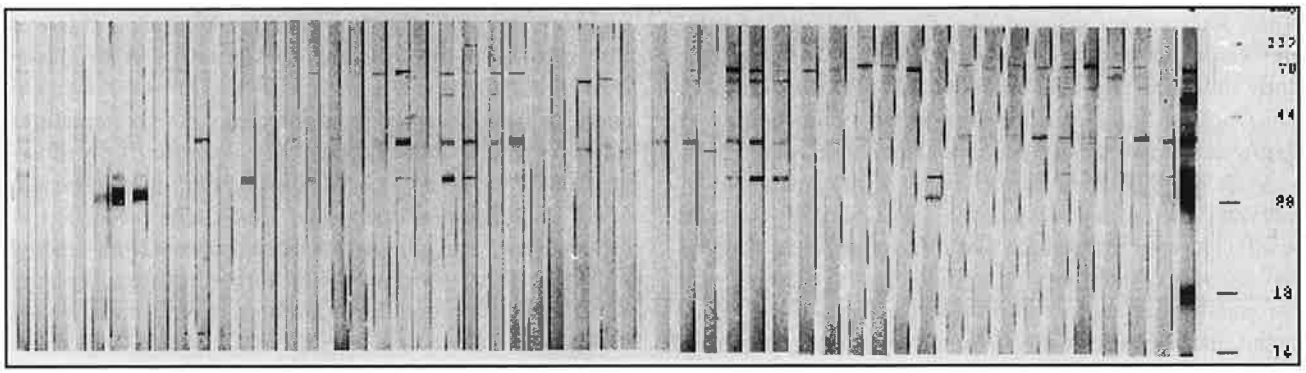

Fig. Western blot analysis of sera from three natural rodent species from Europe: C glareolus, A sylvaticus, *and* A flavicollis. *Immunoreactivity of sera (dil. 1:50) to* B burgdorferi-*specific antigens was tested using whole cell lysates of B. burgdorferi s.s. (ZS 7) as described previously.[40] Most sera recognize antigens of 39-41 kDa and between 60 and 70 kDa. Some sera react with antigens of an approximate molecular mass of 34 to 35 kDa and four sera (8%) show reactivities with antigens of 31 kDa. None of the sera gave signals with antigens of 18-22 kDa.*

Antibodies to proteins of *B burgdorferi ss* of 31 kDa have also been detected in sera from wild *P leucopus* populations in the northeastern United States.[64] The data on the immunoreactivity to *B burgdorferi sl* of sera from wild populations indicate that animals, which were repeatedly infected with spirochetes by ticks, develop additional antibodies to proteins of *B burgdorferi sl.* At present, it is not clear whether antibodies reactive to antigens with molecular masses ranging ~31 kDa are directed to OspA; it is possible that immune sera of naturally infected wild rodents recognize other still undefined proteins of *B burgdorferi sl,* which comigrate in one-dimensional SDS-polyacrylamide gel electrophoreses. The possibility that these antibodies play regulatory roles in the transmission of *B burgdorferi sl* under natural conditions remains to be elucidated.

Although mixed infections in individual reservoir hosts and humans may occur,[65,66] an association between reservoir host species and genomic groups may exist, suggesting that *B burgdorferi sl is* differentiated ecologically.[6] In fact, epidemiological data support this hypothesis and indicate that *B garinii is* transmitted preferentially by birds, whereas *B afzelii is* preferentially transmitted to ticks by rodents.[6,8,31,42]

As *I ricinus* is not host-specific and may feed on different host species during its life cycle, it may acquire different genospecies of *B burgdorferi sl.* Mixed infections in *I ricinus,* including larvae, have, in fact, been recorded.[8] No information is available on whether the different genospecies are preferentially transmitted by *I ricinus.*

Dissemination of *B burgdorferi sl* in tissues and transmission

To establish a systemic infection in a reservoir-competent host, *B burgdorferi sl* has to disseminate from the site of spirochete inoculation. For rodents, it has been reported that it takes between 1 and 4 weeks before an infection becomes systemic,[41,48,53,67] presumably reflecting a rather slow migration of spirochetes in host tissues.

B burgdorferi ss specifically binds host-derived plasmin(ogen), an abundant serum protein with a particular role in fibrinolysis.[68,69] In the presence of specific activators, bound plasminogen is converted into plasmin, which is an aggressive proteolytic enzyme with a broad substrate specificity, eg, it degrades fibrin, fibronectin, and, thus, extracellular matrices. Such bound plasmin(ogen) substantially enhances the migration of *B burgdorferi* through endothelial monolayers.[69] Host-derived bound plasmin also is used effectively by other invasive bacteria and by tumor cells to disseminate in tissues. The plasmin(ogen) system most likely enables *B burgdorferi sl* to migrate from the site of inoculation into adjacent parts of the dermis. As the tick feeding pool is a site of inflammation, cellular infiltration, and wound healing, it is possible that tick saliva affects the conversion of bound plasminogen into its active proteolytic form, thus, influencing spirochete transmission. Recent experiments using mice and sheep provide evidence of rapid spirochete transmission between infected and noninfected cofeeding ticks in the absence of or prior to a systemic infection of the hosts.[48,70]

The interaction of spirochetes and ticks is complex and has recently been reviewed by others.[71,72] The dissemination of spirochetes from the tick's midgut to salivary glands appears to be a prerequisite of spirochete transmission to a host.[26,73-77] Is there a role that plasmin(ogen) plays in the tick-pathogen interaction? It may be speculated that binding and conversion of plasmin(ogen) following the uptake of blood by the tick is a trigger enabling spirochetes to cross the tick's midgut wall and to infect the salivary glands or the ovaries.

TICK-HOST INTERFACE AND TRANSMISSION

Tick-host interaction

In contrast to blood-feeding insects, ixodid ticks feed for a prolonged period of several days on suitable hosts and, thus, are particularly exposed to immune responses of the host. In many cases, natural hosts do not mount an effective response to tick infestations; ticks feeding on such hosts achieve full engorgement and molt to the next developmental stage.[78] It is commonly believed that the absence of an efficient anti-tick immunity is a property of naturally evolved tick-host relationships.[79,80,81] In artificial combinations, such as the New Zealand white rabbit and *I ricinus,* anti-tick immunity (ie, resistance) may be expressed after a few infestations.[82-86] However, certain natural hosts (eg, *C glareolus)* may also acquire an effective immunity to *Ixodes trianguliceps* or *I ricinus* after repeated exposure to ticks.[87, 88] Anti-tick immunity in these natural systems is manifested mainly by a substantially reduced proportion of ticks which engorge fully. Partially engorged ticks do not molt and eventually die a few weeks or months later. In addition, such partially engorged ticks show no tendency to reattach onto a naive host (K.K., unpublished observation, 1995). Acquired resistance in *C glareolus* to *I ricinus* has to be regarded as severe, as it substantially affects the survival rate of ticks.

In *C glareolus,* T cells of the TH-1 subset participate in anti-tick immunity; the release of histamine plays a role as an effector element.[88] Passive transfer of immune serum alone does not confer immunity in naive recipients of the same or other rodent species (K.K., unpublished observation, 1995). In addition, it was found that acquired resistance in *C glareolus* to *I ricinus* occurs frequently under natural conditions in western Europe, regulating the natural tick burden.[18] Most interestingly, in the course of the latter study, individual male, sexually active bank voles were highly infested with *I ricinus* larvae. In such individuals it was not possible to induce a resistance in the laboratory, whereas resistance could be induced consistently in all females of *C glareolus.* This finding indicates a state of immunosuppression possibly associated with hormones such as testosterone.

The expression of resistance to ticks has also been observed in a seminatural avian host of *I ricinus,* the pheasant *(Phasianus* colchicus) (K.K., unpublished observation, 1995). This bird species has been introduced into British woodlands many centuries ago and now serves as an important host for *I ricinus.* In the laboratory, pheasants acquire resistance to *I ricinus* larvae and nymphs after a few tick infestations. Resistance in this avian host kills most of the larvae during feeding, whereas the main effect on nymphs is a reduced engorgement weight. The tick feeding sites of resistant pheasants are characterized by a massive swelling. The immune processes mediating resistance in this host species are unknown.

In contrast to voles or pheasants, mice of the genus *Apodemus* do not develop any resistance to *I trianguliceps*[78] or *I ricinus.*[88] In these rodent species, tick saliva is immunosuppressive and multiple tick bites enhance the feeding success of the ectoparasite.[88] Similar results have been obtained using BALB/c mice.[89, 90]

There is accumulating evidence that the immunosuppression/resistance dynamics in tick-host associations may be substantially modulated by parasite density.[86] The recent findings on resistance and immunosuppression show that the tick infestation level on rodents in nature is not only a function of the density of questing ticks and the host's behavior (eg, home range size), but also determined by the tick-host interaction; the prevalence of acquired resistance in host populations will affect the distribution of immature *I ricinus* within host populations, which is commonly observed to be negatively skewed.

The finding of acquired resistance in natural tick-host associations contradicts an old, but still widely accepted dogma published by Trager in 1939.[79] *C glareolus is* one of the most abundant natural rodent hosts of *I ricinus* or *I trianguliceps* in Europe. This rodent species is more susceptible to various tick-borne pathogens such as the tick-borne encephalitis (TBE) virus,[91] *Babesia microti,*[87] or *B burgdorferi ss,*[40] than members of the genus *Apodemus.* It is possible, therefore, that acquired resistance in this natural tick-host system has been selected for as a defense mechanism against tick-borne pathogens.

Saliva-activated transmission

Events at the arthropod-host interface may have an impact on pathogen transmission. For *Leishmania* spp,[92] TBE virus,[93] and Thogoto virus,[94] it is established that arthropod salivary gland extracts (SGE) enhance transmission or render target cells susceptible to the pathogen, as reported for the protozoon *Theileria parva.*[95] Enhancement of pathogen transmission by arthropod saliva has been termed "saliva-activated transmission" (SAT).[94] For transmission of *B burgdorferi sl,* the role that tick saliva may play in influencing spirochete transmission or modulating the hosts' immune response to spirochetes has not been studied experimentally. There is, however, indirect evidence that tick saliva may enhance spirochete transmission under certain conditions; in mice of the genus *Apodemus,* which were naturally infected with *B burgdorferi sl,* repeated infestation with noninfected *I ricinus* increased the infectivity for ticks.[57] In addition, two recent studies using mice and sheep provide evidence that spirochetes may be transmitted between ticks

feeding in clusters.[48,70] The underlying mechanisms of the increase of spirochete transmission with increasing tick burden are not clear. It is possible that an enhancement of spirochete transmission in mice or sheep is caused by systemic or local immunosuppression mediated by tick saliva. Alternatively, or additionally, tick saliva may enhance the conversion of host-derived plasminogen into plasmin promoting migration of *B burgdorferi* in the skin. The presence or absence of tick saliva may also influence the number of spirochetes required to infect a host, a question that currently is being addressed in the author's laboratory.

Saliva-inhibited transmission

The question arises as to whether the sensitization of a host with tick saliva has any effect on spirochete transmission in those tick-host systems expressing resistance. Wikel and colleagues reported that presensitization of rabbits with ticks reduced tick-borne transmission of *Francisella tularensis,* which was termed "allergic klendusity."[96, 97] In a recent study, the rodents *C glareolus* and *Apodemus* spp. were trapped in biotopes with high and low tick densities described previously.[18] The animals were brought to the laboratory and analyzed for the expression of resistance and for *B burgdorferi sl* infection. Members of the genus *Apodemus* of both biotopes were not resistant to *I ricinus* and about 40% of the rodent population were found to be infected with *B burgdorferi sl.* For *C glareolus* derived from the biotope with low tick densities, very few animals were resistant to *I ricinus* and about 30% of the vole population were infected with spirochetes. All adult *C glareolus* trapped in the biotope with high tick densities, however, were highly resistant to ticks and none of the voles was infected with borreliae (K.K., unpublished observation, 1995).

Based on these findings and on previous laboratory experiments, there is clear evidence that acquired resistance to ticks may block the vector-to-host transmission, whereas the host-to-tick transmission appears to be less affected by resistance.[40,73,98] The mechanism(s) by which such a transmission blockade is mediated is not clear. In view of the fact that in this tick-host system resistance does not kill the ticks while feeding, it is possible that the dissemination of borreliae from the ticks' feeding site into nonaffected skin sites of the host is disrupted by inflammatory immune processes induced by molecules contained in the saliva. Alternatively, or additionally, salivation by ticks (and, thus, spirochete inoculation) may be affected by histamine or other mediators released in response to tick bites.[99] The author proposes to term this kind of transmission blocking immunity "saliva-inhibited transmission" (SIT).

CONCLUSION

Studies using laboratory and natural hosts have shown that antibodies to the outer surface proteins of *B burgdorferi sl* influence spirochete transmission between ticks and hosts. Furthermore, it appears that *B burgdorferi* employs immune evasion strategies to prevent its eradication from the host. The skin is the interface between tick and host where spirochete transmission takes place; recent investigations indicate that events at the tick-host interface also play important roles in transmission. Most of the factors of spirochete transmission discussed in the present paper operate in a density-dependent way. There is emerging evidence that the various tick-host associations differ substantially and that this has profound implications for spirochete transmission.

Studies using laboratory animals have increased our basic knowledge on immune processes and pathogenesis of Lyme borreliosis, whereas the use of natural hosts as animal models has allowed us to estimate accurate parameter values in spirochete transmission .[100]

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The *Borrelia turicatae* Murine Model of Lyme Disease

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ABSTRACT

***Borrelia turicatae* is an agent of relapsing fever. During relapsing fever spirochetes avoid the immune response of the host by a multiphasic antigenic variation. In *Borrelia hermsii*, another agent of relapsing fever, the mechanism for the switch in antigens is a gene rearrangement, namely an interplasmidic gene conversion or an intraplasmidic deletion between direct repeats. In severe combined immunodeficiency (*scid*) mice, *B turicatae* causes the constellation of arthritis, myocarditis, uveitis, and a cranial nerve disorder. In this way, the infection in these mice is similar to Lyme disease. Moreover, *B turicatae* invades and persists in the central nervous system of laboratory mice. The severity of illness, particularly the arthritis, and the entry into the brain appear to be determined by the small Vmp proteins of this species. These proteins are homologous to the polymorphic OspC proteins of *B burgdorferi*, the agent of Lyme disease.**

Key words: *Borrelia*, *Borrelia turicatae*, vertebrate reservoirs

INTRODUCTION

Members of the genus *Borrelia* have in common their transmission by arthropods.[1] Vertebrate reservoirs for these spirochetes are mammals and birds. There are no known free-living *Borrelia* species. With the exception of louse-borne *Borrelia recurrentis* and tick-borne *Borrelia duttoni*, humans are inadvertent hosts for *Borrelia* species. In the cases of Lyme disease and tick-borne relapsing fever in North America, the usual hosts and reservoirs for the borrelias are small mammals. In North America the agent of Lyme disease is *B burgdorferi*. The principal causes of relapsing fever in the United States are the tick-borne species *Borrelia hermsii* in the Northwest and *Borrelia turicatae* in the Southwest.

The association of spirochetes with arthropods of the *Borrelia* species was apparent to investigators earlier in this century.[1,2] Further evidence of the relatedness between *Borrelia* species came with the knowledge of the genetics and biochemistry of these microorganisms. Studies at these levels of organization revealed that other features *Borrelia* species share are linear chromosomes of about one megabase, linear plasmids with hairpin ends, and outer membrane lipoproteins that largely determine the serotype of the cell.[3] The most abundant exposed outer membrane lipoproteins are called Osp proteins in species that cause Lyme disease, including *B burgdorferi*, and Vmp proteins in species that cause relapsing fever, including *B hermsii* and *B turicatae*. The Osp proteins and the Vmp proteins occur in two size ranges. The larger proteins range from 29 kilodaltons (kDa) to 40 kDa. These include OspA, OspB, and OspD in *B burgdorferi* and Vmp7 and Vmp17 of *B hermsii*.[3] The smaller proteins range from 19 to 24 kDa; these include OspC of *B burgdorferi* and Vmp3, Vmp26, and Vmp33 of *B hermsii*.[3] The small proteins of *B burgdorferi* and *B hermsii* are homologous; representative small proteins of each species are approximately 50% identical in amino acid sequence.[4]

Molecular pathogenesis of relapsing fever

Relapsing fever is characterized by 2- to 4-day periods of fever interspersed with 3- to 7-day periods of well-being.[5] At the time of fever there are numerous spirochetes in the blood; between febrile periods few, if any, spirochetes are detectable in the blood. There may be several episodes of spirochetemia before the illness ceases. This has been observed in experimental animals as well as humans.[2,5] After spirochetes have disappeared

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from the blood, they can still be present in the brains of the animals. When immunodeficient *scid* mice are infected with relapsing fever *Borrelia* species the spirochetemia is constant and persistent, an indication that the immune system determines the clearances of spirochetes from the blood (S. Tillman, A.G.B., unpublished data, 1991).

Our studies of the pathogenesis of this disease have concentrated on *B hermsii* and in particular the HS1 strain.[6] The recurrent illness that has been observed during relapsing fever is a result of the multiphasic variation of *B hermsii's* Vmp proteins. These lipoproteins are anchored in the outer membrane by their N-terminal lipid moieties.[7,8] Antibodies to Vmps select against the infecting serotype, which is then replaced in the blood by another serotype. Vmps may function not only as variable antigens that draw the attention of the immune system but may also have a role in the direct interactions of the spirochete with the host; this is discussed in the following.

Each Vmp is coded for by one out of an estimated 40 to 50 *vmp* genes.[4,7,9-12] Only one *vmp* is expressed at a time. This active *vmp is* usually positioned on a 28 kb linear expression plasmid close to a telomere. Silent *vmps* lack a promoter, and in cases of pseudogenes (Ψ*vmp*), part of the coding region.[13,14] They are present either upstream of the expressed *vmp* on the same plasmid or on two silent linear plasmids of 28 and 32 kb.[9]

Activation of new *vmp* gene occurs by transposition of a silent *vmp* to a site downstream of a promoter on the expression plasmid. The new *vmp* replaces the formerly active *vmp*. Activation can occur by at least two different mechanisms. These are shown in Fig 1.

The first is an interplasmidic recombination, in which the source of the silent gene is another plasmid and which is consistent with a gene conversion.[9-11,15] Regions of homology upstream and downstream of the *vmp,* as well as the *vmp* genes themselves, appear to provide the substrates for the recombination. At a variable interval from the 3′ end of the *vmp* gene at the expression site is a 0.2 kb sequence, the Downstream Homology Sequence (DHS), which is repeated in different locations on the silent plasmids, usually just downstream from a *vmp* in those locations. In the interplasmidic switch shown in Fig 1, a serotype 17 cell changes to serotype 4.

The second mechanism is an intraplasmidic recombination, in which the silent *vmp* is adjacent to the expressed *vmp*.[16] In Fig 1, these are *vmp7* and *vmp26*. In this case the silent *vmp26 is* pseudogene *(Ψvmp 26)* lacking the extreme 5′ end of the gene. It is activated by a deletion between short direct repeats in the two *vmp* genes. A product of the recombination is a nonreplicative circle that contains most of the deleted *vmp7* gene.

The activation of some *vmps* by intraplasmidic deletion is followed by an early accumulation of multiple point mutations at the 5′ end of the gene.[14] This is thought to occur by partial gene conversions using silent *vmps* upstream as templates. This phenomenon is remarkably similar to the method used in avian B cells to introduce additional diversity in antibodies.[17]

Differences in disease expression are determined serotype in *B turicatae*

We started our studies of *B turicatae* with the aim of understanding the invasion of the nervous system by spirochetes. Relapsing fever like Lyme disease frequently is complicated by neurologic disorders.[1] We would have preferred to study this neurotropism phenomenon using *B hermsii,* for which we have many reagents and a large database of *Vmp* sequences. However, in past studies[1] and our own work,[18] *B turicatae* was shown capable of invading the mouse brain and persisting there for weeks. *B hermsii* invaded the mouse brain too, but was detectably eliminated from that location even in *scid* mice within 2 to 3 weeks.[19]

In these studies of pathogenesis, we have used immunodeficient *scid* mice. For studies of CNS invasion and tissue localization, immunodeficient mice minimize the risk that neutralizing antibodies will contaminate the preparations or select for new serotypes. Production of antibodies by the mice was assessed by immunofluorescence assays using whole cells.[19] During persistent infection of *scid* mice with *B turicatae* there was variation in the surface proteins the bacteria expressed and in disease manifestations over time.[18] There was no evidence of an antibody response to the borreliae in these animals, and, thus, the variation in the mice apparently reflected selection on the basis of differences in factors other than immunity.

Two serotypes, A and B, were isolated from the mice, cloned by limiting dilution, and further characterized.[18] The only discernible difference between the two variants was in the size of the major surface protein they expressed: serotype A had a *Vmp* of 23 kDa, and serotype B's *Vmp* was 20 kDa A polyacrylamide gel electrophoresis of whole cell lysates of serotypes A and B is shown in Fig 2. Two-dimensional gel electrophoresis of total proteins of the serotypes confirmed that the only difference between the two populations was in the Vmp protein that was expressed (P. Pennington, A.G.B., unpublished data, 1995). Monoclonal antibodies that specifically distinguish between serotype A and serotype B in whole cell immunofluorescence assays bind to VmpA or VmpB in Western blots expressed (P. Pennington, A.G.B., unpublished data, 1995).

When other *scid* mice were inoculated with clonal populations of A and B, the infections were similar with respect to onset and degree of spirochetemia, involvement of the eye and heart, and occurrence of a peripheral

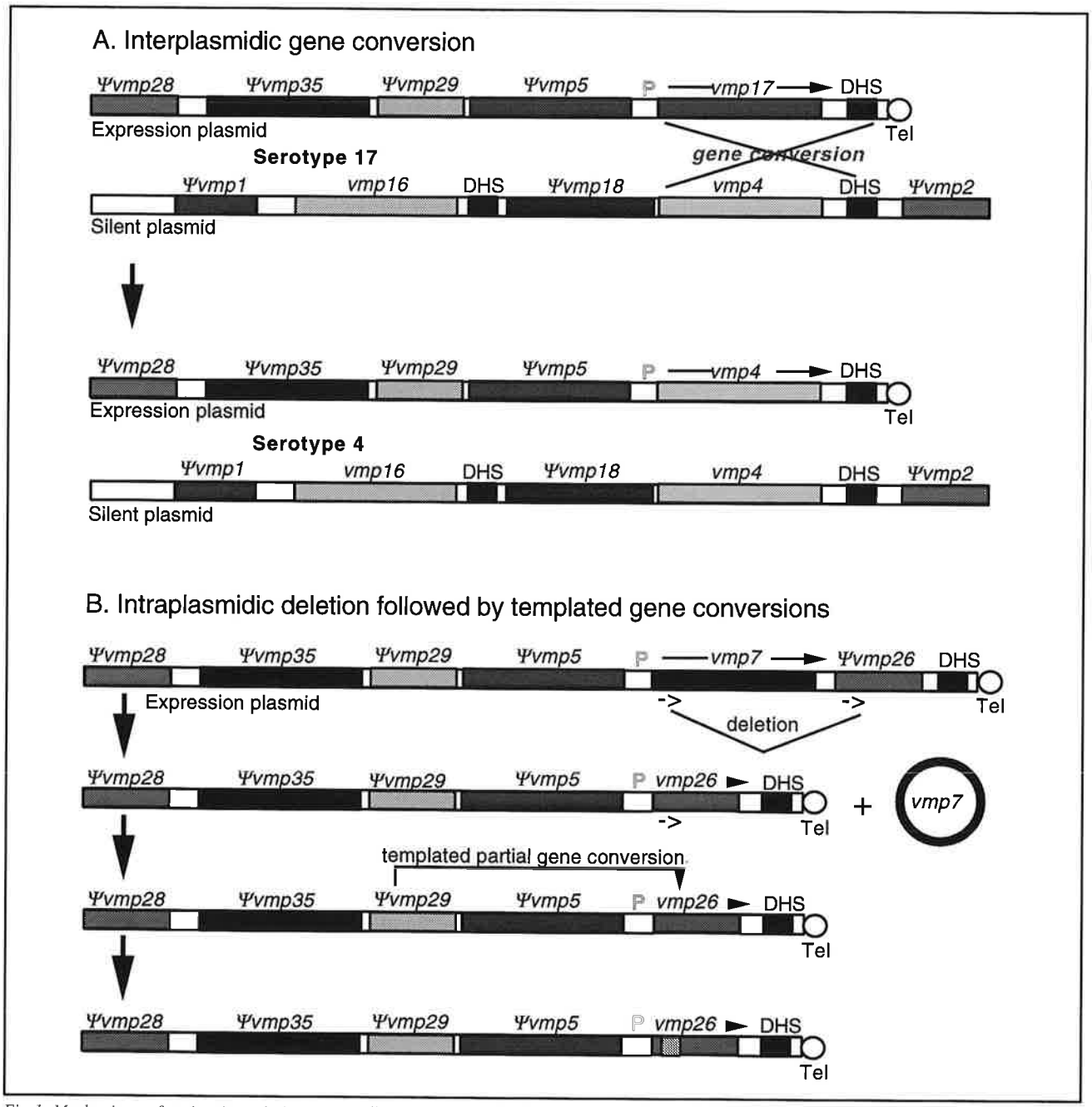

Fig 1. Mechanisms of antigenic variation in Borrelia hermsii. *The right ends of expression and silent linear plasmids are shown. On the expression plasmid the expressed* vmp *gene is adjacent to the Downstream Homology Sequence (DHS) and telomere (tel). The location of the promoter (P) and the direction of transcription (horizontal arrow) are shown. The events depicted are described in the text.*

vestibular disorder. However, there were differences between the serotypes in other respects[18]: (a) Serotype B but not A caused swollen and reddened joints, impaired performance on a walking bar, and severe arthritis by histologic examination. The arthritis was most prominent during the second week of the infection; (b) Serotype A but not B invaded the central nervous system during early infection. Only serotype A could be recovered by culture from the brain or cerebrospinal fluid during early infection. Serotype B could infect and proliferate in the brain if delivered by intracranial inoculation directly but not via the blood; and (c) Serotype A penetrated monolayers of human umbilical vein endothelial cells more readily than did serotype B. The clinical course of infection in mice infected with either serotype A or B is shown in Fig 3.

Both sets of infected mice developed a vestibular dis-

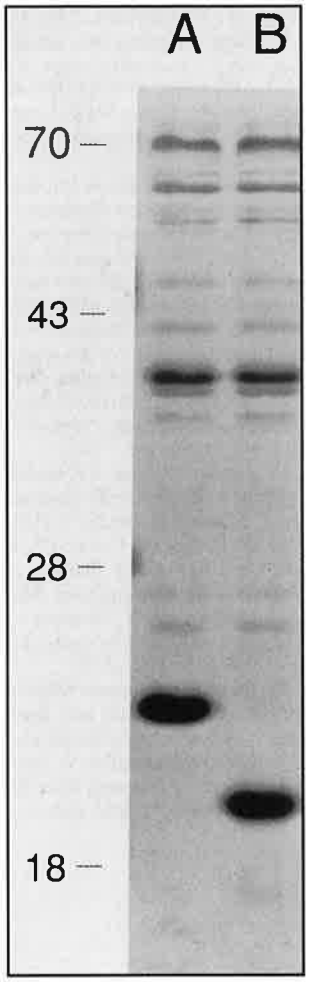

Fig 2. Coomassie blue-stained proteins of whole cell lysates serotypes A and B of Borrelia turicatae *strain Oz1. The acrylamide concentration of the gel was 12.5%. The migrations of molecular weight standards in kilodaltons are shown on the left.*

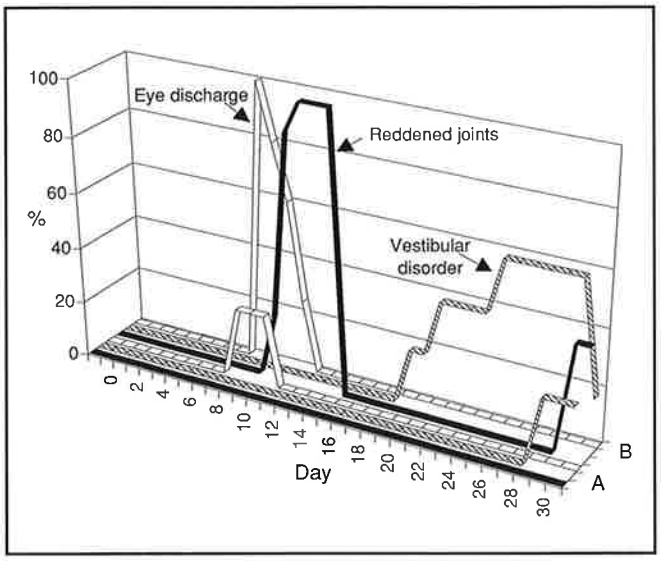

Fig 3. Disease manifestations in scid *mice inoculated with either serotype A (foremost) or serotype B (hindmost) of* B turicatae. *The day of examination is shown at the bottom, and the percentage of mice examined with either mucopurulent eye discharge (white ribbon), joint reddening and enlargement (black ribbon), or vestibular disorder (cross-hatched ribbon) are shown in the y-axis.*
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order, which occurred during the second week of the infection and was more common in serotype B- than in serotype A-infected mice.[18] The neurologic disorder was manifested by head tilt and spinning in the air when lifted off the ground by the tail. The mice also had poor performance on the walking bar at the time of this disorder. The lack of evidence of involvement of the cerebrum or cerebellum on pathological examination indicated that the site of the disease was in cranial nerve VIII. The frequent occurrence of vestibular dysfunction in mice infected with serotype B, which did not invade into brain tissue or cerebrospinal fluid, was other evidence that the disorder was peripheral and not central in origin.

Mice infected with either serotype also had gross purulent discharge of both eyes.[18] This occurred at approximately the same time as the joint swelling. Like the vestibular disorder and severe arthritis, the eye involvement was more common in mice infected with serotype B than in those infected with serotype A. Histopathological examination of eyes of euthanized mice revealed anterior uveitis and conjunctivitis (D. Cadavid and A.G.B., unpublished data). *B turicatae* was present in the eyes of both sets of mice.

The last notable feature of *scid* mice infected with *B turacatae* was a myocarditis.[18] This was characterized by a predominantly mononuclear leukocytic infiltrate between myocardial fibers. The inflammation was greatest around the base of the great vessels and the atria. There was also an epicarditis and pericarditis. Unlike the arthritis, the myocarditis was approximately the same in intensity for serotype A- and B-infected mice.

CONCLUSION

Clearly, *B turicatae* infection of *scid* mice is not equivalent to *B burgdorferi* infection in immunocompetent humans. Although *B turicatae* is related to the agents that cause Lyme disease, it is not the same species. If Lyme disease, especially in its late form, is partly the result of deleterious immune reactions against the host, then the *scid* mouse model will not be very revealing with regard to this aspect of pathogenesis. Nevertheless, there are compelling reasons to study the *B turicatae* murine model. Not the least reason is the constellation of arthritis, myocarditis, uveitis, and cranial nerve disorder that occurs in the majority of infected mice. The persistent infection of the brain by serotype A of *B turicatae* should allow further understanding of the mechanisms by which borreliae enter the central nervous system. An important question for those managing patients with Lyme disease is

the frequency and timing of entry of borreliae into the brain.

The findings to date indicate that differences in disease expression are determined by variable surface proteins of the bacterium. Inasmuch as the small Vmp proteins are homologous to OspC of *B burgdorferi,* there is reason to suspect that polymorphisms of this latter protein may determine disease manifestations in persons and animals infected with *B burgdorferi* as well.

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Safety and Immunogenicity of Recombinant Outer Surface Protein A (OspA) Vaccine Formulations in the Rhesus Monkey

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ABSTRACT

Background: **The safety and the immunogenicity of OspA vaccine formulations were investigated in the rhesus monkey, to help to ascertain possible deleterious effects that could appear both in naive and infected individuals during the vaccination process and especially after a challenge infection had been administered.**

Methods: **Three different vaccine formulations, NS1-OspA/Al(OH)$_3$, a fusion protein composed of recombinant OspA (strain ZS7) lacking the C-terminal cysteine and fused to a fragment of 81 N-terminal amino acids from the non-structural influenza virus protein NS1, NS1-OspA/Al(OH)$_3$/MPL (mono-phosphoryl lipid A), lipidated OspA/Al(OH)$_3$, and one placebo (Al(OH)$_3$) were used in a vaccination trial involving 19 male 2-to-3-year-old Chinese Macaca mulatta. Three 10-µg-doses of each vaccine were given to each animal intramuscularly at 4-week intervals. Group 1 (n=3) was vaccinated after being infected with Borrelia burgdorferi, as a model to assess vaccine safety in patients with an active infection. Group 2 (n=16) was divided into 4 groups of 4, according to the vaccine formulation received. Safety was assessed in both groups of animals by physical examination, clinical laboratory analyses of blood and urine samples, and quantification of inflammation in the anterior eye chamber (uveitis).**

Immunogenicity was assessed by OspA-ELISA, Western blot, antibody-dependent, complement-mediated killing in vitro (ADCK), by the LA$_2$ competitive inhibition assay and by peripheral blood mononuclear cell (PBMC) blastogenesis in vitro in response to OspA.

Results: **No local vaccine site reaction or obvious signs of arthritis were seen in any animal at any time. All urinalyses, serum chemistries, and complete blood count results were either normal or, when not, not attributable to vaccination. No significant intraocular inflammatory responses were found at any time in any animal, thus indicating that the powerful adjuvant properties of lipidated OspA do not cause uveitis. All formulations elicited a strong IgG anti-OspA response, with geometric mean reciprocal titers (GMRT) measured by ELISA as high as 2560 x 10^3, 4 weeks after the last vaccine dose. ADCK$_{50}$ GMRT were as high as 3475 in the lipidated OspA group at 2 weeks before challenge, but declined as much as 15-fold in animals of this group 9 weeks postchallenge. A booster effect on the antibody response was not evident after the challenge infection.**

PBMC blastogenesis was measured longitudinally in vitro, before and after the challenge infection, in response to the mature form of OspA, both lipidated and nonlipidated. PBMC (from animals of all groups except controls) responded to lipidated OspA and the highest responders were among the animals vaccinated with this form of OspA. Responses to nonlipidated OspA were marginal. The PBMC responses to lipidated OspAdeclined to baseline values by 8 weeks after the challenge infection but resurfaced later and remained high until week 32 postchallenge, the last time point determined. No booster effect was observed.

Conclusion: **Within the framework of our study, before postmortem analyses, all vaccine formulations appeared safe, including the lipidated OspA/Al(OH)$_3$ that is currently being used in humans. Immunogenicity at the humoral level was strong but short lived. This, together with the undetectable booster effect, may entail a need for repeated administration of the vaccine.**

Key words: OspA vaccine, rhesus monkey, Lyme borreliosis

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INTRODUCTION

Lyme borreliosis is usually curable with appropriate antibiotics.[1] However, long courses of therapy may be required if the infection is allowed to become chronic, and some patients do not respond to therapy at all.[1] At risk of acquiring a persistent *Borrelia burgdorferi* infection are the 20% to 40% of infected individuals who do not show the telltale erythema migrans.[2] This erythematous papular skin rash is the most important marker of infection, largely because diagnosis based on serology, polymerase chain reaction (PCR), or spirochetal culture, although much improved in the last few years, remains imprecise.[3] Thus, the uncertainty of nonclinical diagnosis of Lyme borreliosis explains in part the occurrence of chronic Lyme disease. The fact that the latter is sometimes refractory to treatment underpins the need for immunoprophylactic strategies.

Recombinant outer surface protein A (OspA) is currently the most promising molecularly-defined vaccine candidate to prevent Lyme borreliosis. Proof of the "principle" that OspA is a protective antigen has been achieved in numerous animal experiments using diverse antigen/adjuvant combinations.[4] More recently, vaccine formulations that are compatible with human use also have been shown to be efficacious in mice.[5-7] Safety and immunogenicity of recombinant OspA vaccines have been evaluated in human subjects with and without a previous history of Lyme disease[8-10] but with no evidence of active infection. In addition, Phase III trials are underway.[11]

The present study of safety and immunogenicity of OspA vaccines in the rhesus monkey was undertaken to ascertain possible deleterious effects that could appear in naive individuals not only during the vaccination process but also after a challenge infection had been administered. Vaccine safety was examined also in a small group of animals that were infected with *B burgdorferi* at the time of vaccination. Immunogenicity was investigated both at the humoral and cellular levels. Because of the relatively rapid decline in anti-OspA antibody titers that had been observed in humans,[8] it was important to assess whether a booster effect was detectable in vaccinated rhesus monkeys subsequent to the challenge infection.

Vaccine formulations with and without added adjuvant were compared with regard to differences in safety and immunogenicity. The animal model was chosen because it had been shown previously that rhesus monkeys infected with *B burgdorferi* develop disease signs that mimic both the acute and chronic phases of Lyme disease in humans. These signs include erythema migrans, arthritis, and neuroborreliosis.[12-14] At the serological level, too, the time course and specificity spectrum of the antibody response to *B burgdorferi* in rhesus monkeys had been shown to be similar to their counterparts in humans.[12] The experimental design used in the present study is described below.

EXPERIMENTAL DESIGN

Distribution of information

All personnel responsible for the clinical examinations, sample collection, vaccine administration, and assays described below were fully informed of the experimental design but were blind to the vaccine formulation or placebo administered to the animals.

Study population

The study population consisted of 19 male 2- to 3-year-old Chinese *Macaca mulatta* (rhesus). Three of these animals, L379, L452, and L453 (Group 1), were infected with *B burgdorferi* 4 months prior to vaccination, as a model to assess safety of vaccination in previously infected patients. The 16 other animals, L457, L549, M021, M581, L458, L594, L971, M585, M243, L537, M219, M107, L476, L712, L642, and M106 (Group 2), were divided into four groups of four animals each, according to the vaccine formulation received.

Animal examination schedule

During and after vaccination, animals of Group 1 were examined on a weekly to biweekly basis. Animals of Group 2 were examined biweekly during the 12-week period before the challenge infection, and weekly thereafter.

Vaccine formulations

Three different vaccine formulations and one placebo were used:

1) NS1-OspA/$Al(OH)_3$, a fusion protein adsorbed onto aluminum hydroxide. The NS1-OspA fusion protein is composed of recombinant OspA (cloned from the *B burgdorferi sensu stricto* isolate ZS7) that lacks the carboxyl-terminal cysteine and is fused to a fragment of 81 N-terminal amino acids from the nonstructural influenza virus protein NS1. This formulation was given to animals L458, L594, L971, and M585;

2) NS1-OspA/MPL/$AlOH)_3$, as above, but combined with 50 µg of the adjuvant (immune modulator) 4′ - monophosphoryl lipid A (MPL). This formulation was given to animals L476, L712, L642, and M106;

3) lipidated OspA/$Al(OH)_3$, recombinant lipidated OspA (ZS7) adsorbed onto aluminum hydroxide. This formulation was given to animals M243, L537, M219, and M107.

4) Aluminum hydroxide: was used as a placebo and was given to animals L457, L549, M021, and M581. All

vaccine formulations contained 10 μg/ml OspA, 0.5 mg/ml of $Al(OH)_3$, and 5 mg/ml of 2-phenoxyethanol (a preservative) in a buffer containing 150 mM sodium chloride, 5 mM sodium phosphate, and 5 mM potassium phosphate, pH 6.8.

Vaccine administration protocol

All vaccines/placebos were administered by intramuscular injection into the cranial thigh, alternating left and right thighs with each injection. Three 10-μg doses of the chosen vaccine formulation were given to each animal at 4-week intervals. The animals in Group 2 were given a challenge infection 4 weeks after the last vaccine dose. The three animals in Group 1 were vaccinated 4 months after being infected with *B burgdorferi*, using the same vaccination regimen as the animals of Group 2. The vaccine formulation chosen for Group 1 was NS1-OspA/MPL/$Al(OH)_3$.

Inoculation with *B burgdorferi*

All animals were inoculated with the B31 strain of *B burgdorferi* by the natural route of tick bite using infected *Ixodes scapularis* nymphal ticks. The procedure used and the source of ticks were the same as those described previously.[12]

Evidence that animals of Group 1 were infected

After 5 days of exposure to 20 ticks each, four ticks had fed upon animal L379, 11 upon L452, and two upon L453. All ticks were shown to be infected with *B burgdorferi* by direct immunofluorescence with a *B burgdorferi*-specific antibody, a procedure described previously.[15] Clinical examinations performed weekly on all animals revealed a mild-to-moderate skin erythema peripheral to the inoculation site in animal L452 by week 1 postinoculation (PI), which lasted for a total of 2 weeks. Animal L379 showed a mild erythema in the same site 2 weeks PI. Histological assessment of skin biopsies obtained weekly revealed deep perivascular lymphocytic infiltrates considered characteristic of the dermatitis associated with human erythema migrans in all animals until week 4 PI. By week 6 PI, the dermatitis was no longer present. Immunohistochemistry performed on these same skin sections using a monoclonal antibody to a 7.5 kDa lipoprotein of *B burgdorferi*[12] showed that *B burgdorferi* antigens were present both within and outside dermal macrophages in sections from all animals obtained after weeks 1 and 2 PI. Biopsies taken prior to infection were normal. Corneal biopsies obtained from all animals showed a marked conjunctivitis microscopically by week 2 PI, and immunostained positively for *B burgdorferi*. Fractions of skin sections from all animals—processed for PCR analysis as described previously[12]—contained detectable *B burgdorferi* DNA by week 3 PI. No spirochetes were cultured in vitro from skin or blood samples collected during the first 4 weeks PI. Western blots of sodium dodecyl sulfate (SDS)-solubilized B31 spirochetes were developed with anti-IgM and IgG antibodies. The IgM response peaked by weeks 2 to 3 PI and gradually waned. In contrast, the number of antigens recognized by anti-*B burgdorferi* IgG antibodies increased gradually over time, an indication of an active infection. By week 4 PI, four to six antigens were recognized, depending on the animal; by week 17 PI, (1 week after the last vaccine dose had been administered), 9 to 12 antigens; and by week 44 PI, 12 to 18. Prominent amongst the antigens recognized were P41 (flagellin) and P39.

Assessment of vaccine safety

Safety was assessed in both groups of animals (1 and 2) by physical examination of the animals, clinical laboratory analyses of blood and urine samples, and quantification of inflammation in the anterior eye chamber. Physical examination involved general observation, thoracic auscultation, determination of body temperature and weight, and gauging of lymph node and spleen sizes by palpation. Joints were examined for presence of swelling or redness. Laboratory analyses included complete blood cell count, serum chemistries, and urinalysis. Ocular inflammation was assessed both using the slit lamp and by laser flare photometry.

Assessment of vaccine immunogenicity

Vaccine immunogenicity was longitudinally assessed in animals of Group 2, both at the humoral and cellular levels. Antibody responses were assessed qualitatively by Western blot of serum samples using SDS extracts of whole *B burgdorferi* B31 as antigen; appearance of both IgM and IgG antibodies was examined. The overall IgG antibody response was quantified by ELISA, using purified recombinant OspA without the NS1 residue (MDP-OspA) as antigen. MDP-OspA also lacks the lipid moiety but contains the tripeptide Met-Asp-Pro covalently attached to the lysine in position 18 of the native OspA molecule. Bactericidal antibody was quantified by antibody-dependent, complement-mediated killing of *B burgdorferi* in vitro (ADCK) and functional, protective antibody by the LA_2 assay, a competitive inhibition assay in which the anti-LA_2 epitope antibody present in a serum sample is quantified by competition with the binding of the LA_2 monoclonal antibody, an antibody that binds to the LA_2 functional epitope of OspA. Cellular immune responses were examined by blastogenesis of peripheral blood mononuclear cells (PBMC), measured in vitro in response to 1) the T-cell mitogen concanavalin A (Con A), as a positive control; 2) recombinant MDP-OspA

from *B burgdorferi* strain ZS7; and 3) recombinant lipidated OspA from the same strain.

MATERIALS AND METHODS

Animal care and housing

Animals were cared for and housed as described previously.[12]

Western blotting procedure

The procedure used was described previously[16] but *B burgdorferi* strain B31 (4th passage) was used rather than strain JD1.

Enzyme-linked immunosorbent assay (ELISA)

Plates were coated with 1.2 μg/ml MDP OspA (100 μl per well) in 0.1 M carbonate buffer, pH 9.6. Plates were incubated with antigen O/N at 4°C and washed three times with 200 μl/well of phosphate-buffered saline (PBS) containing 0.1% Tween 20 (PBS/T1). Plates were blocked for 2 h at 37°C with 200 μl of 1.5% BSA, 1.5% nonfat dry milk in PBS/T1 per well. A volume of 100 μl of test serum appropriately diluted with PBS/T1 containing 0.75% BSA, 0.75% nonfat dry milk was added to each well and incubated for 1 h at 37°C. Horse-radish peroxidase-labeled goat anti-human IgG (γ-chain specific) (Kirkegaard and Perry Lab Inc, Gaithersburg, Md) was diluted to 1:2000 and 100 μl were added to each well and incubated for 1 h at 37°C. The color development reagent (3,3′,5,5′-tetramethylbenzidine) was used according to the manufacturer's instructions (Kirkegaard and Perry Lab Inc, Gaithersburg, Md). All samples, including the preimmune serum, were titered by serial dilution. Titer is defined as the maximum dilution whose optical density (OD) is $\geq$ to 3 x OD obtained with the same dilution of the preimmune serum of the same animal.

Antibody-dependent, complement-mediated killing of *B burgdorferi* in vitro

The ADCK assay was performed as described. All determinations for each animal and time point were performed in duplicate. The mean percentage killing of these duplicate determinations was plotted as a function of the serum dilution, and the serum dilution corresponding to 50% killing ($ADCK_{50}$) was obtained from each titration curve by interpolation.

LA_2 inhibition assay

The LA_2 competitive inhibition assay was performed as follows: 96 well plates (Immunoplate Maxisorp, Gibco, Grand Island, NY) were coated with purified recombinant lipidated OspA by incubating the plates overnight at 4°C with 100 μl/well of a 0.5 μg/ml solution of OspA in 0.05 M $NaHCO_3$ buffer, pH 9.6. The optimal coating concentration of OspA was determined for each batch of purified protein. Unbound OspA was removed by washing four times with a 0.15 M NaCl, 0.05% Tween 20 solution and the plates were then "blocked" with 200 μl/well of PBS 1% bovine serum albumin for 30 min at room temperature, and washed again as above. Monkey serum samples and the LA_2 antibody standard solution were diluted twofold serially, starting at a dilution of 1:2 or 1:10, and a concentration of 4 μg/ml, respectively. Dilutions were made with PBS 0.2% BSA containing 0.05% Tween 20. A volume of 100 μl of each dilution was added to each of duplicate wells and left for 2 h at 37°C. Plates were then washed as above and incubated for 2 h at room temperature, with 100 μl/well of a limiting concentration of LA_2 antibody labeled with horseradish peroxidase (1/10,000 for the antibody batch used) in PBS 0.2% BSA, 0.05% Tween 20. After washing as before, a volume of 100 μl/well of 0.4 mg/ml o-phenylenediamine (Sigma Chemical Company, St. Louis, Mo), 0.15% H_2O_2 in a 0.1 M citrate buffer, pH 4.5 was added. The reaction was stopped after 15 min by adding 50 μl/well of 1.0 N HCl, and the OD were read at 490 nm in a microplate reader. The raw data were analyzed by the four-parameters method. The results are expressed in μg/ml of LA_2 equivalent, using data from the standard LA_2 monoclonal antibody curve as a reference for interpolation.

Analysis of significance

Significance of the differences between mean antibody titers (ELISA, ADCK, or LA_2) elicited by, and laser flare photometry values in groups receiving, different vaccine formulations was established by analysis of variance.

Peripheral blood mononuclear cell blastogenesis

Antigens and mitogens. For each blastogenesis assay, MDP-OspA and lipidated OspA were used at 1, 5, and 10 μg/ml. The concentration of antigen eliciting the maximal response at each time point was used in the data analysis. Con A (Sigma) at 8 μg/ml was the optimal concentration chosen based on a preliminary dose-response analysis.

Culture conditions for in vitro blastogenesis. Blood was obtained from each animal and mixed with preservative-free heparin during extraction. PBMC were obtained by the Ficoll-Hypaque (Sigma) density gradient centrifugation method. The final viable cell counts were adjusted to 2 x 10^6/ml in RPMI-1640 medium (Cellgro, Fisher Scientific, Pittsburgh, Pa) containing 10% heat-inactivated human AB serum (Sigma). The blastogenesis assays were performed in triplicate in round (antigen cultures) or flat bottom (mitogen) microtiter plates (Costar Corporation, Cambridge, Mass). To each well, 100 μl of antigen or mitogen and 100 μl of cell suspension were

added. Cultures were incubated at 37°C in a humidified atmosphere (5% CO_2 and 95% air) for 4 days (mitogen) and 6 days (antigen). Approximately 24 h before harvesting, 1.0 μCi of [^{3}H] thymidine (ICN, Irvine, Calif) was added to each well. The cells were harvested onto glass-fiber mats, washed and dried overnight at room temperature. The dried filters were placed into 5 ml of scintillation fluid, and radioactive incorporation was measured in a liquid scintillation counter. Results are expressed as stimulation index (SI), ie, counts per minute (CPM) of stimulated cells divided by CPM of unstimulated cells. The cut off SIs were 4.15 and 5.13 for MDP OspA and lipidated OspA, respectively, which represent the mean SI for all animals before vaccination began, plus 2 SD for each antigen.

Anesthesia protocol

All animals were anesthetized with ketamine-HCl (10 mg/kg) given by intramuscular injection. Animals known to have excessive myoclonic activity under ketamine anesthesia were anesthetized with tiletamine and zolazepam in combination (Telazol 10 mg/kg) by intramuscular injection. If necessary, injectable anesthesia was supplemented with 1% to 3% isoflurane and O_2. Analgesia was provided after skin biopsies with butorphanol tartrate (0.05 mg/kg).

Hematology, serum chemistries, and urinalyses

Complete blood count and serum chemistry determinations as well as urinalyses were all standard.[17] Serum chemistry analyses included tests for serum electrolytes, glucose, alanine aminotransferase, serum alkaline phosphatase, aspartate aminotransferase, lactate dehydrogenase, blood urea nitrogen, creatinine, globulin, and albumin.

Slit-lamp examinations and laser flare photometry

Following external inspection of conjunctiva and adnexa, slit lamp and laser flare photometry (LFP) examinations were performed in animals of both Group 1 and 2 at baseline and at 1 and 2 weeks following each of the three immunizations. Animals of Group 2 were also examined at 1, 2, and 3 weeks following the challenge infection. LFP was performed as described elsewhere,[18] using a KOWA laser flare meter (KOWA, Inc., Torrence, Calif).

RESULTS

Vaccine safety

Physical examination: No local vaccine site reaction was noted in any animal (Groups 1 and 2) at any time. In animals of Group 2, a transient splenomegaly was observed in 3 of the 4 animals that were given $Al(OH)_3$ alone at 2 weeks after the challenge infection (PC) and in one of the animals that received lipidated OspA/$Al(OH)_3$ at 3 weeks PC. Clinical signs of arthritis were not seen in any animal. Body temperatures were taken initially at 2-week intervals coinciding with physical examinations; no fever was noted. To determine whether vaccination would cause fever shortly after the time of injection, body temperature was measured 1 day after the application of the third injection in the animals of Group 2, and 1 day after the second and third injections in the animals of Group 1. Some of the animals from Group 2 (uninfected) demonstrated a temperature marginally above the baseline for rhesus monkeys (>102.0 °F) one day after vaccination. These animals were uniformly distributed among all vaccination groups, including that receiving $Al(OH)_3$ alone. None of the animals of Group 1 (preinfected) demonstrated a temperature one day after vaccination. Therefore, these febrile episodes cannot be associated with the effects of OspA in any of the forms employed, including the lipidated form.

Urinalysis: All urinalysis samples were within normal limits in all animals.

Complete blood count: An initial leukocytosis was seen in all animals prior to experimental manipulation and in some cases also thereafter. This is a common phenomenon when animals kept in outdoor breeding groups are moved to indoor single cage housing for project work, and is probably a physiologic response to stress. In most cases seen at the Tulane Primate Center this leukocytosis resolves within 2 weeks. The leukocytosis was mild to moderate in intensity and could not be associated with any particular vaccination protocol in so far as it occurred with similar frequency in all vaccination groups, including that receiving $Al(OH)_3$ alone.

Serum chemistry: With two exceptions, all animals were within normal values for all the serum enzymes tested. The exceptions were animals M585 (Group 2), vaccinated with NS1-OspA/$Al(OH)_3$ and L712 (Group 2), vaccinated with NS1-OspA/MPL/$Al(OH)_3$. Animal M585 showed marginally elevated alanine aminotransferase (ALT>100 units) after the third vaccine dose and at the time of tick removal. Animal 712 also showed elevated ALT at the time of tick removal (198 units) and both elevated ALT and aspartate aminotransferase (AST) (AST>100 units) at 1, 2, and 4 weeks PC. AST and ALT are hepatocellular enzymes which, when elevated, indicate hepatocellular death or increased hepatocyte cell membrane permeability.

Quantitative assessment of inflammation in the anterior eye chamber

Animals were examined at the slit lamp and by LFP; LFP is a procedure which objectively measures intraocular inflammation.[18-20] A pilot study was performed to

TABLE 1

QUANTITATIVE ASSESSMENT OF INFLAMMATION IN THE ANTERIOR EYE CHAMBER USING LASER FLARE PHOTOMETRY (LFP).

Vaccination Group	Time of observations	LFP (Mean ± SD)
Baseline Normals n = 40 eyes.	NA	Mean 3.9 ± 0.5
Group 1 n = 6 eyes	Baseline 6 eyes flare	3.4 ± 1.2
	Baseline post-infection	4.7 ± 2. 4
	Baseline prior to V1,V2	3.9 ± 2.1
	Flare @ I wk post-V1,V2	3.8 ± 1.9
	Flare @ 2 wks post-V1,V2	3.6 ± 2.1
Placebo [Al(OH)$_3$] n = 8 eyes	Baseline -Flare	3.4 ± 1.8
	I wk post-V1,V2,V3	3.7 ± 1.7
	2 wks post-V1,V2,V3	3.8 ± 1.8
	Challenge baseline	3.9 ± 1.3
	I wk PC	3.6 ± 1.5
	2 wks PC	2.9 ± 1.0
	3 wks PC	3.5 ± 1.4
NS1-OspA/Al(OH)$_3$ n = 8 eyes	Baseline -Flare	2.9 ± 1.2
	I wk post-V1,V2,V3	3.3 ± 1.2
	2 wks post-V1,V2,V3	3.5 ± 1.5
	Challenge baseline	3.1 ± 1.6
	I wk PC	2.5 ± 0.9
	2 wks PC	3.1 ± 1.2
	3 wks PC	2.8 ± 1.0
NS1-OspA/MPL/Al(OH)$_3$ n = 8 eyes	Baseline -Flare	3.3 ± 1.5
	I wk post-V1,V2,V3	3.6 ± 1.9
	2 wks post-V1,V2,V3	3.5 ± 1.5
	Challenge baseline	3.7 ± 1.5
	I wk PC	2.8 ± 0.9
	2 wks PC	3.3 ± 1.2
	3 wks PC	3.3 ± 0.5
Lipidated OspA/Al(OH)$_3$ n = 8 eyes	Baseline -Flare	3.7 ± 1.0
	I wk post-V1,V2,V3	3.5 ± 1.7
	2 wks post-V1,V2,V3	3.7 ± 1.6
	Challenge baseline	3.3 ± 1.3
	I wk PC	3.0 ± 1.0
	2 wks PC	3.3 ± 0.7.
	3 wks PC	3.2 ± 0.7

V = vaccination 1,2,3.
PC = post-challenge.

establish the reliability of the laser cell flare meter in the rhesus monkey. In this study, the results of baseline and follow-up examinations by slit lamp and LFP were compared in 10 animals, to establish the correlation between a subjective and objective examination method. Inflammatory responses were induced surgically in the right eyes by paracentesis of the anterior chamber. A comparison of LFP baseline readings of the inflamed right eyes and simultaneous readings of the untreated contralateral left eyes documented a significant fibrinous inflammatory response (P=0.008) within the right eyes. This correlated well with the slit lamp findings (subjective). Based on these results, photometry data collected during and after the vaccination procedure could be assumed to be reliable.

Rhesus monkeys that received the different vaccine formulations and were subsequently challenged (Group 2) and the animals that were vaccinated after infection (Group 1) were examined at baseline and at 1 and 2 weeks following each of the three immunizations. Animals of Group 2 were also examined at 1, 2, and 3 weeks following the challenge infection. These results are shown in Table 1. Additionally, 40 eyes of normal rhesus monkeys were examined to establish baseline values of normal anterior chamber LFP.

In previous vaccine studies using the simian immunodeficiency virus model, the LFP value of 5.0 was established as the upper limit for normal, uninflamed eyes (M.D. Conway, MD, unpublished data). In the present study, this value was never reached. LFP values from the vaccinated animals or from animals within the "immunopotentiation" Group 1 did not differ significantly from baseline data within each group or from photometry values obtained with the independent group of normal animals. All LFP evaluations were simultaneously confirmed by slit lamp examinations. Therefore, no significant intraocular inflammatory response was found at any time within the framework of this study.

Vaccine immunogenicity at the humoral level

Western blot analysis: Western blots were performed using serum samples from the 16 animals in Group 2 at 2 weeks after the first vaccine/placebo injection and 4 weeks after the second injection. The results are shown in Figs 1A and 1B, respectively. By the second week after the first injection, an antibody response to OspA was already detectable in most animals that had received OspA. The intensity of the OspA bands depended on the vaccine formulation used, and increased in the sequence NS1-OspA/Al(OH)$_3$, NS1-OspA/MPL/Al(OH)$_3$, lipidated OspA/Al(OH)$_3$. This was true of both the IgM and the IgG responses, although it was more marked for the IgG response. Control animals showed no response to OspA (Fig 1A). By week 4 after the second injection, the banding pattern of the anti-OspA IgG antibody response became very complex (Fig 1B). Antibodies reacted with antigens

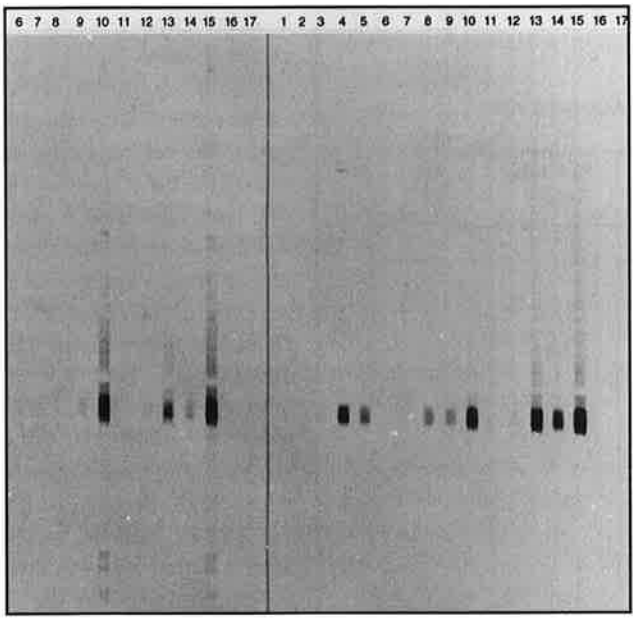

Fig 1A. Serum samples collected 2 weeks after the first immunization.

Pooled serum taken before the first injection from all animals was used as negative control (track 1), and monoclonal anti-OspA antibody H5332 as positive control (track 18). Serum samples from the 4 animals that received placebo are on tracks 2, 6, 11, and 16, respectively; NS1-OspA/Al(OH)$_3$ on tracks 3, 7, 10, and 17; NS1-OspA/MPL/Al(OH)$_3$ on tracks 4, 8, 9, and 12; lipidated OspA/Al(OH)$_3$ on tracks 5, 13, 14, and 15. IgM antibodies are shown on the left-hand panel and IgG antibodies on the right-hand panel. Western blot was done as described in Materials and Methods.

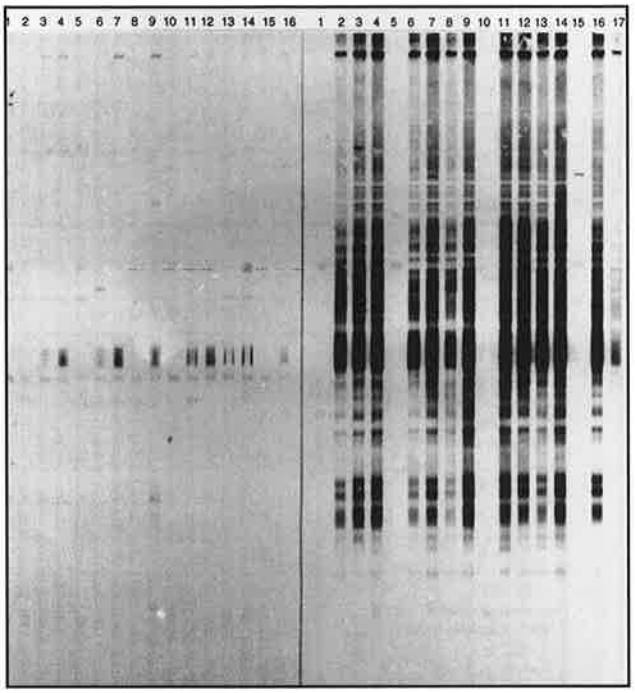

Fig 1B. Serum samples collected 4 weeks after the second immunization.

Monoclonal anti-OspA antibody H5332 was used as positive control (track 17). Serum samples from the 4 animals that received placebo are on tracks 1, 5, 10, and 15, respectively; NS1-OspA/Al(OH)$_3$ on tracks 2, 6, 9, and 16; NS1-OspA/MPL/Al(OH)$_3$ on tracks 3, 7, 8, and 11; lipidated OspA/Al(OH)$_3$ on tracks 4, 12, 13, and 14. IgM antibodies are shown on the left-hand panel and IgG antibodies on the right-hand panel. Western blot was done as described in Materials and Methods.

of molecular weights that were both higher and lower than the 31 kDa B31 OspA. This band is indicated by monoclonal anti-OspA antibody H-5332 (Fig 1B, track 17). The IgM antibody response to OspA contrasted with the IgG response in that it did not evolve the complex banding pattern of the IgG response and appeared to subside 4 weeks after the second injection, as compared with the level observed 2 weeks after this time (not shown). On occasions, serum samples from animals that had received placebo reacted with antigens in the higher molecular weight region (eg, track 15, Fig 1B). Most of this reactivity disappeared when serum samples were used at a dilution of 1:200 (not shown) rather than the 1:50 dilution used in the experiments described above. In contrast, reactivity patterns of serum samples from animals vaccinated with OspA did not change noticeably when used at a dilution of 1:200.

Enzyme-linked immunosorbent assay: Anti-OspA IgG antibody titers were determined by ELISA throughout the vaccination process. Serum samples were titrated on weeks 2, 4, 6, 8, 10, 12, and 21 after the first injection, ie, 2 and 4 weeks after the first injection, 2 and 4 weeks after the second injection, 2 and 4 weeks after the third injection, and 9 weeks PC, respectively. The results, expressed as geometric mean reciprocal titers (GMRT) per "vaccination group" are shown in Fig 2, together with the titer range per group. IgG anti-OspA antibody titers increased as a function of time, and reached remarkably high values, in the range of $1:10^6$, regardless of the immunization protocol. By the end of the vaccination procedure, the group that had received the lipidated form of OspA had achieved the highest GMRT of IgG anti-OspA antibody. However, differences between groups were not significant at the 95% level. After the last injection, titers appeared to decline rapidly, for by week 13 after the last injection (9 weeks PC), the GMRT had decreased 14-fold in the NS1-OspA/Al(OH)$_3$ group, 38-fold in the NS1-OspA/MPL/Al(OH)$_3$ group, and 10-fold in the lipidated OspA/Al(OH)$_3$ group.

Antibody-dependent, complement-mediated killing and LA_2 assays: $ADCK_{50}$ titers were determined with serum samples collected on weeks 4, 8, and 10 after the first injection and LA_2 titers only with the latter samples. The results are shown in Table 2. As expected, $ADCK_{50}$ values increased as a function of time during the vaccination process. Both the $ADCK_{50}$ and LA_2 mean values by

TABLE 2

ADCK$_{50}$ AT WEEKS 4, 8, AND 10 AFTER THE FIRST VACCINE DOSE AND LA$_2$ VALUES AT WEEK 10.

Vaccine Formulation /Animal #	ADCK$_{50}$			LA$_2$ (μg eq./ml)	ADCK$_{50}$ GMRT	LA$_2$ GMT
Placebo	**Wk 4**	**Wk 8**	**Wk 10**	**Wk 10**	**Wk10**	**Wk10**
L457	NA	NA	NA	0.3		
L549	NA	NA	NA	0.3		
M021	NA	NA	NA	0.4	NA	0.3
M581	NA	NA	NA	0.2		
NS1 OspA Al(OH)$_3$						
L458	NA	320	1000	49.9		
L594	NA	300	675	122.1		
L971	15	225	750	136.4	854	104
M585	NA	ND	1050	141.1		
NS1 OspA MPL Al(OH)$_3$						
L476	15	1800	2250	115.6		
L712	NA	60	525	97		
L642	NA	750	4200	399.1	2173	172
M106	NA	ND	4500	193.4		
Lipidated OspA Al(OH)$_3$						
M243	18	ND	9000	556		
L537	NA	1800	9000	793		
M219	105	ND	480	115	3475	376
M107	105	ND	3750	396		

NA: not applicable (serum sample does not contain sufficient antibody to promote 50% killing under the conditions in which the ADCK experiment is performed.

ND: Not determined.

week 10 after the first inoculation were significantly higher (95% level) in animals that had received the lipidated form of OspA and Al(OH)$_3$, compared to the group that had received NS1-OspA/Al(OH)$_3$. Other differences between groups were not significant at this time. In selected animals, the ADCK$_{50}$ titer was determined at the time of challenge (week 12 after the first injection) and 9 weeks PC (Table 3). As with the ELISA, a sharp decline in the titer was noted in all animals. Animal L537 (lipidated OspA/Al[OH]$_3$), which had an ADCK$_{50}$ titer of 15 000 at the time of challenge, had a titer of only 960, a 15-fold decrease, 9 weeks later. Animal L712 (NS1-OspA/ MPL/Al[OH]$_3$) experienced a 10-fold decrease in titer (from 700 to 70) in the same time interval, and animals L476 (NS1-OspA/ MPL/Al[OH]$_3$) and M585 NS1-OspA/Al[OH]$_3$, a four-fold decrease.

Vaccine immunogenicity at the cellular level

Immunogenicity at the cellular level was investigated by quantifying in vitro the blastogenic responses of PBMC when these cells were stimulated either with recombinant nonlipidated OspA (MDP-OspA) or recombinant lipidated OspA, both from B burgdorferi strain ZS7. The results of a longitudinal study including samples taken at 4, 8, 13, 23, 27, 32, 36, 40, and 44 weeks after the first vaccine dose are summarized in Fig 3. Each time point represents the mean stimulation index (SI) of the four animals in each vaccination group. The error bar represents the standard error of the mean. The SI for each animal is itself the mean value of a triplicate determination. The time of each vaccine injection is indicated on the horizontal axis by each of the first three arrows. The challenge infection occurred 12 weeks after the first injection (fourth arrow in Fig 3). The antigens used for blastogenic stimulation are listed at the bottom of the figure. The horizontal dotted line represents the mean value of the SIs of all animals measured before vaccination, in response to each of the two antigens used, plus 2 times the value of the standard deviation of each mean.

Based on this criterion, the cut-off values were 4.15 and 5.13 for MDP-OspA and lipidated OspA, respectively. PBMCs from the placebo group did not respond to any antigen at any time. Regardless of the vaccination protocol, the response to the nonlipidated (MDP) form of OspA was minimal. In contrast, the response to lipidated OspA was significant in all "vaccine" groups at some time during the vaccination protocol or after the challenge infection. At 15 weeks after the first vaccine dose (3 weeks PC), the mean SI values were 7.7, 17.3, and 37.2 in the NS1-OspA/Al(OH)$_3$, the NS1-OspA/MPL/Al(OH)$_3$, and

lipidated OspA/Al(OH)$_3$ groups, respectively. However, the responses declined to background levels within the first 11 weeks PC (Fig 3).

Interestingly, after this time the mean PBMC response of the animals that had received lipidated OspA increased again, and oscillated around an SI of 16 through the last time point measured. PBMC responses to the T-cell mitogen Con A were also measured for each animal at the same time points, as a positive control for T cell responsiveness. The mean SI values for each vaccine/placebo group and the corresponding SI range are shown in Table 4, for cells obtained 4, 8, 15, and 27 weeks after the first vaccine dose. Throughout this time period (and also thereafter) the PBMCs of all animals continued to show a vigorous response to Con A.

TABLE 3

CHANGE IN ADCK$_{50}$ WITH TIME: ADCK$_{50}$ IN SELECTED ANIMALS AT WEEKS 10 AND 12 AFTER THE FIRST VACCINE DOSE, AND 9 WEEKS POST-CHALLENGE.

Animal #	Vaccine Formulation	ADCK$_{50}$		
		Wk 10	Wk 12	Wk 9 PC
L457	Al(OH)$_3$	NA	NA	20
M021	Al(OH)$_3$	NA	NA	15
L712	NS1-OspA/ MPL/Al(OH)$_3$	525	700	70
L476	NS1-OspA/ MPL/Al(OH)$_3$	2250	2800	720
M585	NS1-OspA/ Al(OH)$_3$	1050	600	135
L537	Lip OspA/ Al(OH)$_3$	9000	15000	960

TABLE 4

MEAN PBMC RESPONSES TO CON A OF ANIMALS THAT RECEIVED THE SAME VACCINE TYPE, AT DIFFERENT TIMES AFTER THE FIRST VACCINE DOSE.

Vaccine Type	Mean SI Range			
	wk 4	wk8	wk15	Wk 27
Al(OH)$_3$ Placebo	520 96 - 1107	568 93 - 1237	409 71 - 982	372 72 - 693
NS1-OspA Al(OH)$_3$	432 281 - 595	617 364 - 1163	609 257 - 1374	366 193 - 506
NS1-OspA MPL/Al(OH)$_3$	451 69 - 382	537 333 - 728	329 74 - 490	199 61 - 365
Lipidated OspA/ Al(OH)$_3$	375 306 - 451	1089 580 - 1439	676 365 - 863	432 327 - 522

DISCUSSION

We investigated the safety and the immunogenicity of three recombinant OspA vaccine formulations in the rhesus monkey. The advantage of performing such a study in an animal model is that it is possible to examine the effects which a challenge or a pre-existing active infection might have on both the safety and the immunogenicity of a vaccination protocol. Postmortem analyses are also possible in animals. The disadvantage, of course, is that in animal models the disease syndrome, the immune response to the infection and to the vaccine, and the interplay between vaccine and infection may differ from that found in humans. With our choice of model we sought to minimize this disadvantage, for both the acute and chronic phases of the Lyme disease syndrome are well mimicked in the rhesus monkey infected with *B burgdorferi*.[12-14] As it turned out, the results of two safety and immunogenicity studies performed in humans[8,9] are comparable to those obtained in the rhesus monkey. In the human studies, vaccine safety was evaluated clinically by recording adverse systemic (fever, headache, malaise, arthralgia) or local (pain, redness, swelling, induration) signs and symptoms observed during the vaccination procedure [8,9] and up to 6 months after the last vaccine dose.[8] In one of the studies, standard urinalyses and hematologic and serum chemistry analyses were performed as well.[9]

Both studies showed no significant adverse effects other than transient local pain and tenderness at the site of inoculation. In our study, no local vaccine site reaction was noted in any animal, before or after the challenge infection (Group 2), or when the vaccine was given to preinfected animals (Group 1). Therefore, all vaccine formulations were locally nonreactogenic in the rhesus monkey, as assessed clinically. Systemically, both control and vaccinated Group 2 animals occasionally exhibited fever,

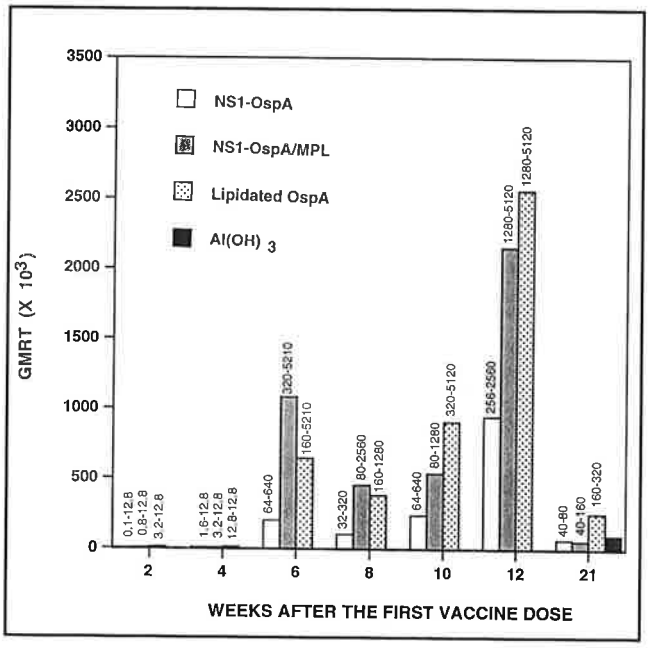

Fig 2. Geometric mean reciprocal antibody titers of serum obtained during and after the vaccination period.

Geometric mean reciprocal titers (GMRT) were determined by ELISA using MDP-OspA as antigen, as described in Materials and Methods. The GMRT range for each vaccination group at each time point is depicted on top of the corresponding histogram bar. Week 21 after the first vaccine dose corresponds to week 9 PC.

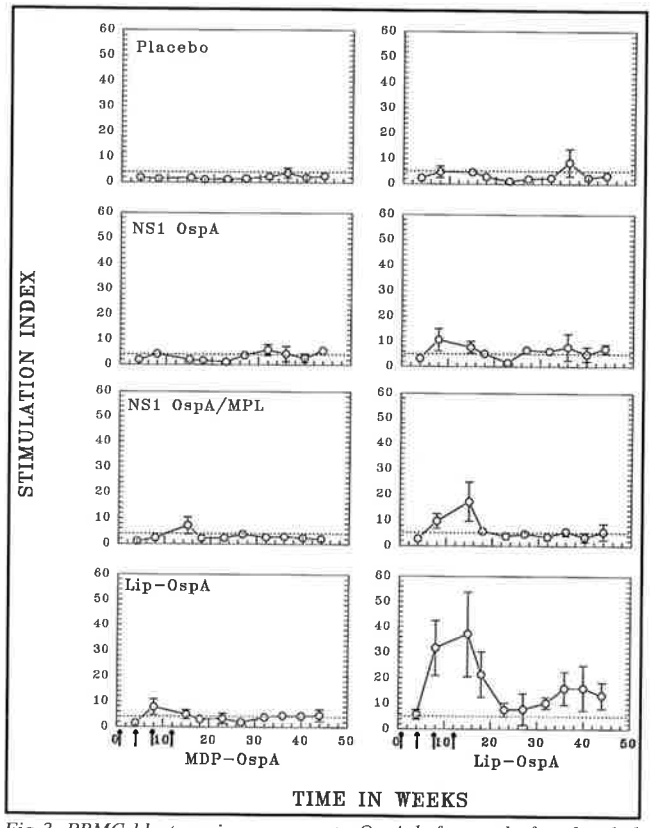

Fig 3. PBMC blastogenic responses to OspA before and after the challenge infection.

Each time point represents the mean stimulation index (SI) of PBMC from the four animals in each vaccination group. The error bar represents the standard error of the mean. The SI for each animal is itself the mean value of a triplicate determination. The time of each vaccine injection is indicated on the horizontal axis by each of the first three arrows. The challenge infection occurred 12 weeks after the first injection (fourth arrow). The antigens used for blastogenic stimulation, non-lipidated OspA (MDP-OspA) and lipidated OspA (L-OspA), are listed at the bottom of the figure. The horizontal dotted line used as cut-off value represents the mean value of the SIs of all animals measured before vaccination, in response to each of the two antigens used, plus two times the value of the standard deviation of each mean.

so this sign cannot be ascribed unequivocally to the vaccine. Clinical signs of arthritis were not seen in any animal at any time. As with humans, no evidence of alterations in medullar or renal functions could be gleaned from the hematologic and urine analyses. Two animals that had received NS1-OspA/Al(OH)$_3$ and NS1-OspA/MPL/Al(OH)$_3$, respectively, showed elevated hepatic enzyme levels, on one occasion before, and on several occasions after the challenge infection. Because changes were transient and mild, their etiology is unlikely to be a toxic effect attributable to the vaccine.

One of the key issues pertaining to vaccine safety is whether an adjuvant such as MPL or a highly immunogenic molecule such as the lipidated form of OspA could promote uveitis.[21,22] The mitogenic and cytokine-producing properties of the lipidated form of OspA have been analyzed recently in vitro using mouse splenocytes and bone marrow-derived macrophages.[23] As little as 5 to 10 ng/ml of lipidated OspA elicited proliferation of splenocytes of naive mice and induced the secretion of interleukin-6, tumor necrosis factor-α, γ-interferon, and nitric oxide in macrophages. In contrast, the nonlipidated form of the molecule was inactive in these assays.[23] Moreover, the lipidated form of OspA, but not the unlipidated homolog, was shown to deliver augmenting costimulatory signals for the activation of T lymphocytes.[24] These results, and the fact that the antibody response produced in mice by the lipidated form of OspA is significantly higher than that induced by the nonlipidated molecule and is directed chiefly to peptide epitopes,[25-27] indicate that the lipid moiety on the OspA molecule acts as a potent adjuvant.[23]

Historically, vaccines containing derivatives of muramyl dipeptide have been implicated in the induction of uveitis and the breakdown of the blood ocular barrier when administered to rabbits.[21] The use of these compounds in vaccine preparations has created concern, especially after the appearance of uveitis in animals that had received muramyl dipeptide derivatives or other adjuvants.[21,22,28] Uveitis production would be a drawback for any candidate vaccine since, once the blood ocular barrier is broken, it may be compromised more easily by subse-

quent nonselective inflammatory events.

Nutrients and metabolites necessary to sustain intraocular structures are supplied by the aqueous humor in the eye. Protection of the delicate nervous tissue is provided by unique tight junctions between the endothelial cells of the ciliary body. This endothelium provides a blood-ocular barrier that normally controls the selective permeability of plasma components and liposoluble substances into the aqueous. If the barrier is damaged by inflammation, protein infiltration increases, raising the concentration of protein and leukocytes in the aqueous humor.[29] Damage to the blood-ocular barrier is usually evaluated subjectively by slit-lamp examination, during which a light beam is narrowed and placed into the center of the pupil to estimate the protein content of the aqueous (flare) and the number of red and white blood cells that appear in the aqueous, or objectively by LFP.

Our evaluations of the animals by slit lamp and LFP indicated that no inflammatory response or permeability change of the blood-ocular barrier was experienced by any animal, either during the vaccination process of preinfected or naive animals, or after the challenge infection. Thus, within the framework of this study, neither the immunomodulator MPL nor the lipidated form of OspA, with its strong adjuvant properties, caused uveitis. Because a similar study was not performed in humans, a comparison is not possible. We expect, however, that in view of the uniformity of our results, uveitis may be either clinically insignificant or may not arise at all.

Our analysis of the antibody response by Western blot indicates that the vaccination protocols employed do not elicit a strong or long-lasting IgM response to OspA. The anti-OspA IgM antibody also did not evolve the complex banding pattern of the IgG response. This banding pattern appearing at molecular masses that are both higher and lower than that of OspA is intriguing, for it is antigen-specific: addition of increasing amounts of soluble recombinant OspA to serum samples prior to their incubation with the nitrocellulose strips, gradually eliminated all the bands by competitive inhibition; the 31 kDa OspA band was the last to disappear (unpublished data). The simultaneous occurrence of SDS-resistant OspA aggregates and OspA degradation products seems unlikely. Alternatively, antibody specificities elicited during the affinity maturation process of the anti-OspA response could bind with low(er) affinity to OspA epitopes shared by other *B burgdorferi* proteins. Although it is reasonable to assume that the lipid moiety is a target for this cross-reactivity, the complex banding pattern was elicited with both lipidated and unlipidated OspA and, moreover, it is very similar to that exhibited by the anti-OspA monoclonal antibody H-5332 used as a positive control. This monoclonal antibody is known to react with a peptide portion of the OspA molecule.[30] A better explanation for its causes notwithstanding, the "OspA" banding pattern is a fact that may complicate the detection of seroconversion to anti-*B burgdorferi*-antibody-positive by Western blot in patients participating in vaccine efficacy trials. In our own study of efficacy of the OspA vaccine in the rhesus monkey (unpublished data) we solved this problem using the competitive inhibition described previously.

In the study in which the same vaccine formulations that were used by us were employed,[9] the highest "functional" (LA_2) anti-OspA antibody titer was achieved with the lipidated form of OspA. Four weeks after the administration of the last vaccine dose, this GMT was about 40-fold the baseline value that is regarded as protective, about two-fold that elicited by NS1-OspA/MPL/$Al(OH)_3$, and six-fold that obtained without MPL. This trend was mimicked in our study regardless of whether functional antibody titers were measured by ADCK or by the LA_2 assay. However, the geometric mean LA_2 titer of functional anti-OspA antibody and also the ELISA titers, were much higher in monkeys than in humans, eg, the LA_2 GMT elicited by the lipidated form of OspA and $Al(OH)_3$ was 86 times higher in monkeys at a comparable time during the vaccination procedure.[9] In this sense then, the antibody response to the OspA vaccine in the rhesus model differs from the human immune response. This divergence is not only of obvious importance when evaluating efficacy of the OspA vaccine in the rhesus monkey but may also be relevant in the present study, for if there were immunopathological signs associated with high titers of anti-OspA antibodies, as has been suggested in view of the correlation found between IgG antibody reactivity to OspA and treatment-resistant arthritis,[31] these signs might be readily apparent in the rhesus model. Our present study shows no such evidence, but a more complete picture will be obtained after joint tissues are examined postmortem.

The bactericidal antibody response to OspA was transient both in humans and in monkeys. In humans, the bactericidal anti-OspA antibody titer was down to baseline values 20 weeks after the last vaccine dose.[8] In monkeys, it had not reached baseline values 13 weeks after the last vaccine dose but it had decreased between four-fold and 15-fold. Because the challenge infection occurred 9 weeks before this time (4 weeks after the last vaccine dose), it follows that the infection could have elicited only a transient booster effect, and probably none at all. The absence of a detectable booster effect is probably due to the mode of action of the OspA vaccine, to the peculiar expression pattern of the OspA molecule, or to both. The absence of a booster effect could have arisen from the annihilation of

the spirochetal population within the tick midgut,[32] or the depletion of this population to very low (nonimmunogenic) levels. Furthermore, recent work by de Silva and colleagues[33] (reviewed by Barthold[34]) has demonstrated that OspA is no longer detectable on the spirochetal surface (and probably ceases to be expressed altogether) after the organisms reach the tick salivary glands, immediately before infection. Hence, spirochetes that survived the onslaught of antibody to OspA in the midgut would be unable, all the same, to boost the immune response to this antigen. From a practical standpoint, this entails that it may be necessary to administer OspA vaccines repeatedly on a scheduled basis, so as to maintain bactericidal antibody titers at an effective level.

Like the anti-OspA antibody response, the cellular response to OspA also appeared to be transient. Although blastogenic responses of PBMC were evident by week 8 after the first vaccine dose in all vaccination groups when the cells were stimulated in vitro with lipidated OspA, 4 weeks PC (week 16 after the first vaccine dose) the response appeared to decline, and reached background levels 10 weeks PC. Interestingly, however, the response to lipidated OspA started to climb again by week 32 after the first vaccine dose, and oscillated around an SI of 16 through the last time point measured. Taking into consideration the resurfacing of this response and that T cells within the PBMC compartment continued to respond to Con A throughout the study period, it appears as though the response to lipidated OspA was specifically and temporarily suppressed rather then intrinsically short-lived.

Although speculative, it is possible that a transient *B burgdorferi* infection may have induced the down-regulation of the PBMC response. The subsequent elimination of this putative infection allowed the response to reappear. A tick-induced modulation of the immune response also may have contributed to this phenomenon.[35] Throughout this study, lipidated OspA elicited a stronger blastogenic response than MDP-OspA in all vaccination groups. This was not due to the reported mitogenicity of this molecule,[23] as PBMC from the animals that received placebo did not respond to lipidated OspA and, moreover, stimulation indices were measured after 6 days of incubation with antigen, when mitogenic responses have long been elicited. Rather, it is possible that the reported ability of lipidated OspA to elicit the B7.1 costimulatory pathway[24] may be the cause of this phenomenon.

It was recently reported that T cell lines isolated from PBMC or synovial fluid of human Lyme disease patients with treatment-resistant arthritis recognized OspA significantly more frequently than T cell lines from patients with treatment-responsive Lyme arthritis. This prompted the concern that exposure to *B burgdorferi* after vaccination with OspA, as in our Group 2, or vaccination of a person with an undiagnosed (active) infection, as with Group 1, might induce immunopathologic T cell responses affecting the joints.[36] Postmortem examination of the joints of the animals will allow us to address this concern. Our results thus far do not justify it.

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Dissemination of *Borrelia burgdorferi* After Experimental Infection in Dogs

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ABSTRACT

The dissemination of *Borrelia burgdorferi* in six dogs 3 to 4 months after exposure to *B burgdorferi*-infected ticks was examined by spirochete isolation in culture medium and polymerase chain reaction (PCR). *B burgdorferi* were identified most frequently from the following tissues: skin, synovial membranes, peritoneum, pericardium, fascias, skeletal muscles, lymph nodes, and meninges. In contrast, blood, spleen, kidneys, urinary bladder, urine, brain, and CSF were negative. The spirochetes appear to have a predilection for connective tissues in dogs. Culture and PCR were both positive in 32 specimens; in addition, 13 were positive in culture and 10 were positive by PCR only. *B burgdorferi* isolation or PCR from skin biopsy samples near the site of the tick bite was found to be reliable for identifying *B burgdorferi* infection in dogs.

Key words: Canine, Lyme disease, *Borrelia burgdorferi*, PCR, culture, tissue dissemination

INTRODUCTION

Lyme borreliosis is a tick-transmitted disease of humans, dogs, and other animals, caused by the spirochete *Borrelia burgdorferi*.[1-3] The spirochetes are transmitted by ticks, including *Ixodes scapularis* in the eastern and north central United States of America,[2,4] *Ixodes pacificus* in California and Oregon,[4] *Ixodes ricinus* in Europe,[5] *Ixodes persulcatus* in East Europe and Asia,[6,7] and *Ixodes ovatus* in Japan.[8] Lyme disease is a multisystem disorder in humans,[9] depending partially on the *Borrelia* species.[3,10,11] In addition to joint disease, it has been suggested that dogs may develop neurologic,[12] cardiac,[13,14] or renal disease[12-14] after natural infection. The main clinical signs from natural infection in dogs are arthritis and arthralgia.[13-16] The mechanisms underlying the pathologic changes accompanying canine and human Lyme disease remain to be defined.

To investigate the pathogenesis of Lyme disease in dogs and to facilitate the evaluation of the efficiency of a vaccine or antibiotic treatment of Lyme disease, we compared spirochete isolation in BSK-II medium with polymerase chain reaction (PCR) for an accurate diagnosis of Lyme infection in dogs. Detection of *B burgdorferi* directly by in vitro culture or PCR has been reported in humans[17-21] and animals.[22-32] These techniques are also useful for examination of *B burgdorferi* in skin and tissue samples of dogs.[22]

In this study, we report the dissemination of *B burgdorferi* in SPF dog tissues as established by isolator in culture and compared with PCR. The possible reasons for the persistent infection by *B burgdorferi* are discussed.

METHODS

Dogs

Six 6-week-old specific pathogen-free (SPF) beagles from the James A. Baker Institute for Animal Health, Cornell University colony were used. Dogs were fed a commercial ration and water *ad libitum*. All dogs were observed daily for clinical signs and daily body temperature was recorded. Dogs were euthanized 3 to 4 months after tick exposure. Three dogs (No. 2, 3, 4) were lame at

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Table 1
Culture and PCR of **B burgdorferi** *From Tissues of Six Dogs 3 to 4 Months After Tick Challenge*

	Dog # (Culture /PCR)					
Tissues*	1	2	3	4	5	6
Skin	+/+	+/+	+/+	+/+	+/-	+/+
Synovial membranes						
Left shoulder	-/-	+/+	+/-	-/-	-/-	-/-
Right shoulder	-/-	+/+	+/-	+/+	-/-	-/-
Left stifle	-/-	+/+	-/-	+/+	-/-	-/-
Right stifle	+/-	-/+	-/-	-/-	-/-	-/-
Left elbow	-/+	+/+	+/+	-/-	-/-	-/-
Right elbow	-/-	-/-	-/-	-/-	-/-	-/+
Lymph nodes						
Left superficial cervical	-/-	+/+	-/-	+/+	-/-	-/-
Right superficial cervical	-/-	+/+	-/+	+/+	-/-	-/+
Left axillary	+/+	+/-	+/+	-/-	-/+	-/-
Right axillary	+/+	+/+	+/+	-/-	-/+	-/-
Left popliteal	-/-	-/-	-/-	-/-	-/-	-/-
Right popliteal	-/-	-/-	-/-	-/-	-/-	-/-
Fascias						
Left front (antebrachial)	-/-	+/+	-/-	-/-	-/+	-/-
Right front (antebrachial)	-/-	+/-	+/-	+/-	-/-	-/-
Left hind (lata)	-/-	-/-	-/-	-/-	-/-	-/-
Right hind (lata)	-/-	-/-	-/-	-/-	-/-	-/-
Skeletal muscles						
Left fore limb (triceps brachii)	-/-	+/+	+/+	-/+	-/-	-/-
Right fore limb (triceps brachii)	-/-	+/+	+/-	+/+	-/-	-/-
Left hind limb (adductor)	-/-	-/-	-/-	-/-	-/-	-/-
Right hind limb (adductor)	-/-	-/-	-/-	-/-	-/-	-/-
Myocardium	+/-	-/-	-/-	-/-	-/-	-/-
Pericardium	+/+	+/+	-/-	+/+	+/-	-/-
Peritoneum	+/+	+/-	-/-	-/+	-/-	-/-
Meninges (dura mater)	-/-	-/-	+/-	-/-	-/-	-/+

**The following tissues were negative by both PCR and culture in all six dogs (CSF, urine, blood, kidney, spleen, uvea, adrenal gland, brain stem, cerebrum, cerebellum, urinary bladder, and achilles tendons taken from hind legs).*

the time of necropsy and three dogs (No. 1, 5, 6) appeared clinically normal.

Ticks

Adult ticks (*I scapularis)* were collected by dragging white flags in a forested area in North Salem, Westchester County, New York. Ticks were maintained at 94% relative humidity at 10°C until used. For determining the percentage of ticks infected with *B burgdorferi,* 20 ticks were cultured individually as previously described.[22] Sixty percent of the ticks were infected with *B burgdorferi.*

Dog exposure to ticks

Fifteen female and 7 male adult ticks were placed onto the clipped right side of each dog as described.[22] After 7 days, the ticks were collected and counted, and the level of engorgement was determined. At least 50% of the female ticks were fully engorged.

Isolation of *B burgdorferi*

For attempts to isolate *B burgdorferi* from blood, CSF, or urine at the termination of the study, 1 mL of sterile heparinized blood taken from the jugular vein, 1 mL of sterile urine taken from the urinary bladder, and 0.5 mL of CSF were cultured in BSK-II medium as previously described.[22] Skin biopsies near the site of the tick bite were obtained using a 4-mm skin puncture at monthly intervals after tick exposure and at necropsy. They were ground in BSK-II medium with a pellet pestle and placed into 6.5 ml of medium. Tissues taken at post-mortem (see Table 1) were homogenized using a tissue homogenizer (stomacher: Tekmar, Cincinnati, Ohio) and cultured in BSK-II medium as previously described.[22,33] The medium was incubated at 34°C for 6 weeks and examined weekly by darkfield microscopy for the presence of live spirochetes. IFA staining with rabbit anti-*B burgdorferi* was used to identify the spirochetes as *B burgdorferi,* if observed in the culture medium.

Polymerase chain reaction (PCR)

The DNA from biopsy samples (skin) or from post-mortem tissues (Table 1) was extracted by a standard procedure.[22,26,27,34] Also, 25 tissues including synovial membranes, lymph nodes, muscles, peritoneum, pericardium, and skins collected from each of the two SPF dogs were used as a negative control. The DNA from *B burgdorferi* was isolated as previously described.[22,26,27,34] PCR was performed as described[22,26,27,34] using three primers derived from the *B burgdorferi* 41 kDa flagellin gene. Primer 1 (5′ATTAACGCTGCTAATCTTAGT-3′), primer 2(5′-CAAAATGTAAGAACAGCTGAA-3′), and primer 3 (5′-GTACTATTCTTTATAGATTC-3′) were synthesized with an Applied Biosystems 380A DNA Synthesizer (Foster City, Calif) from the Analytical and Synthetic Facility, Cornell University.

To prevent contamination, the preparation of reaction mixtures, the DNA extraction, the amplification, and detection of the PCR products were performed in different rooms. Also, aerosol-resistant filter pipette tips were used throughout the experiment. The amplification of *B burgdorferi* flagellin-specific target sequences was carried out in a 50-μL reaction mixture containing 100 ng of DNA from the specimens listed in Table 1 or 1 ng of *B burgdorferi* genomic DNA for positive control or distilled water for negative control, 50 mM KC1, 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 0.5% NP40, 0.5% Tween 20, 200 μm each deoxynucleoside triphosphates, 2 μm of primer sets 1/3, or 2/3 and 2 U of the thermostable *Taq* DNA polymerase (Perkin-Elmer Cetus). The reaction mixture was overlaid with 100 μL mineral oil to prevent evaporation and was subjected to 40 cycles of amplification by using an automated DNA thermal cycler (Perkin-Elmer Cetus, Foster City, Calif). Each cycle involved heating to 94°C for 1 min (DNA denaturation), cooling to 39°C for 1 min (primer annealing), and heating to 72°C for 2 min (primer extension). Negative controls were included in each PCR run which consisted of distilled water substituted for the DNA template in the reaction mixture.

For nested PCR, the first 40 cycles were performed with primer 1/3 under conditions described above. After the last cycles, 2 μL of the reaction mixture was added to a new PCR mixture containing 30 pmol of primer set 2/3, which is directed against the internal portion of the 1/3 amplification product, and was again subjected to an additional 40 cycles. Each cycle for the second reaction in the nested PCR involved heating at 94°C, annealing at 55°C, and extension at 72°C.

Visualization of the PCR amplification products was performed by gel electrophoresis on a 6% polyacrylamide gel with pBH20-*HinfI* cut as a size marker. For Southern blot analysis, the PCR amplification product was run on a 1.5% agarose gel, stained by ethidium bromide, denatured in denature buffer (1.5 M NaCl, 0.5 M NaOH) for 1 hr, neutralized in neutralization buffer (1 M Tris-HC1 pH 8.0, 1.5 M NaCl), and transferred to a nitrocellulose membrane as previously described.[35] The oligonucleotide (primer 2) was 3′ labeled with a nonradioactive labeling kit (ECL 3′-oligolabelling system, Amersham, Bittle Chalfont, Buckinghamshire, England). Southern blot hybridization and detection were performed as described by the manufacturer.

Kinetics-ELISA and immunoblots

The Kinetics-ELISA (KELA) for measuring levels of serum antibody to *B burgdorferi* was described previously.[22,33,36] Immunoblot analysis was performed in a miniblotter as previously described.[22,33,36]

RESULTS

Clinical sign

All six dogs showed an intermittent elevation of body temperature for one or two days after exposure to infected ticks. Three dogs showed one or two brief episodes of lameness (right front limb) between three and four months after exposure, when they were euthanized. The other three dogs appeared clinically normal at the time of necropsy.

Distribution of *B burgdorferi* in dog tissues by culture and PCR

Tissues (Table 1), as well as blood, urine, and CSF taken at necropsy from the six dogs were cultured and PCR was performed. All of the tissues from the two SPF dogs were negative by using PCR technique. The follow-

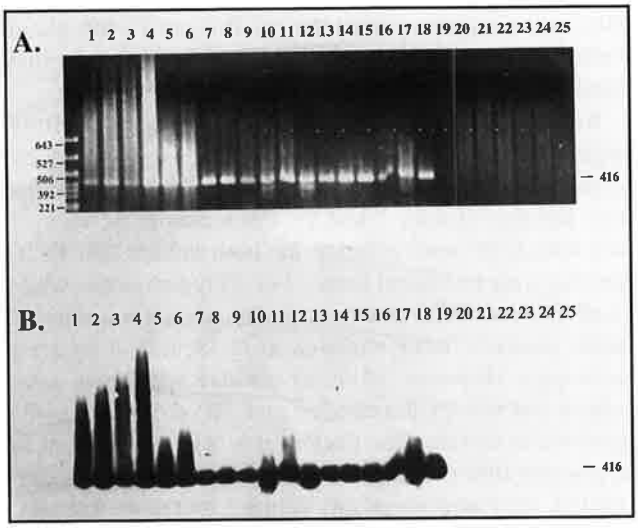

Fig 1. A: An agarose gel showing representative PCR results by using tissue from infected dogs. Template DNA (100 ng) was extracted from joint capsules(lanes 1-5), lymph node (lanes 6-8), fascia (lane 9), muscles (lane 10-11), pericardium (lane 12), and skin (lanes 13-17) from dogs 1-4 and 6, respectively, positive control (lane 18, 1 ng of B burgdorferi *genomic DNA was used as a template), negative control (lane 19, instead of a DNA template, distilled water was used), and CSF (lane 20), urine (lane 21), blood (lane 22), spleen (lane 23), kidney (lane 24), and brain stem (lane 25). Except skin samples, all tissues were from dog 2. B: Southern blot hybridization representative PCR products from A. The technique used was described before Methods.*

ing tissues contained *B burgdorferi* in cultures from at least one of the dogs: skeletal muscle, synovium of the stifle, shoulder and elbow joint capsules, antebracheal fascias, peritoneum, myocardium, pericardium, axillary and superficial cervical lymph nodes, and skin. No organisms were cultured from the: spleen, adrenal gland, urinary bladder, right and left tendons of hind legs, brain stem, cerebrum and cerebellum, uvea, CSF, urine, blood, or kidney (Table 1). The total number of culture/PCR positive tissues from three lame dogs was 37 versus 18 from three clinically normal dogs. PCR results were very similar to isolation results in culture (Tables 1 and 2). DNA gel electrophoresis (Fig 1A) and DNA hybridization results showed the PCR products to bind primer 2 (Fig 1B).

KELA and immunoblot

All dogs showed 300 to 400 slope units of KELA activity by 48 days after exposure to infected ticks (Fig 2). Sera were negative at the time of exposure. Sera with >100 slope units are considered to be positive.[33,36,37] Western blot analysis showed a strong immune response to whole cell antigens 48 days after tick exposure (data not shown).

DISCUSSION

In this study of experimental Lyme disease in the dog, we have examined by concurrent culture and PCR the dissemination of *B burgdorferi* in tissues 3 to 4 months after infection. Skin biopsies near the site of the tick exposure, taken during the course of the 3 to 4 month study, were positive by culture and PCR. They are useful for the clinical diagnosis of Lyme disease in the dog[22,38] and man.[19,20,39-41] Attempts to isolate *B burgdorferi* from dog blood were not done in this study because with few exceptions, they were negative in an earlier study.[22] At the time of euthanasia, blood samples, urine, CSF, synovial membranes, lymph nodes, skeletal muscles, fascial samples, peritoneum, pericardium, and meninges, as well as most major organs, were sampled for culture and PCR (Table 1). All six dogs gave negative results by both techniques in blood samples, spleen, kidney, urinary bladder, urine, CSF, and brain as well as adrenal gland and uvea. In contrast, *B burgdorferi* were found by culture in tissues taken from skin, synovial membranes, lymph nodes, fascias from front limbs, front limb skeletal muscles, pericardium, and peritoneum. *B burgdorferi* was also isolated from the myocardium of one dog, meninges of one dog, and DNA was detected in another (Table 1). Interestingly, twice as many tissues taken from lame dogs were culture/PCR positive in comparison to tissues taken from clinically normal dogs. In addition, the fact that most isolates were made from frontal tissues closer to the site of the tick attachment would speak against a hematogenous spread of the *B burgdorferi.*

There appears to be a considerable difference in the dissemination of *B burgdorferi* in rodents on one hand and in dogs and perhaps in humans and rhesus monkeys on the other. *B burgdorferi* are isolated routinely from the spleen, kidneys, and urinary bladder of rodents[23-27,29,30,42-48] but these tissues are rarely infected in humans or dogs.[22] The spirochetes in dogs were predominantly found in connective tissues such as synovial membranes, fascias, skeletal muscles, peritoneum, and pericardium.

Table 2
Comparison of the Number of Culture and PCR Positive Tissues in Different Organs of Six B burgdorferi *Infected Dogs*

	(Number positive/number tested)	
	Isolation	**PCR**
Skin	6/6	5/6
Joint capsules	10/36	10/36
Lymph nodes	12/36	14/36
Fascia	4/24	2/24
Skeletal muscles	5/24	5/24
Heart	1/6	0/6
Pericardium	4/6	3/6
Peritoneum	2/6	2/6
Meninges (Dura mater)	1/6	1/6

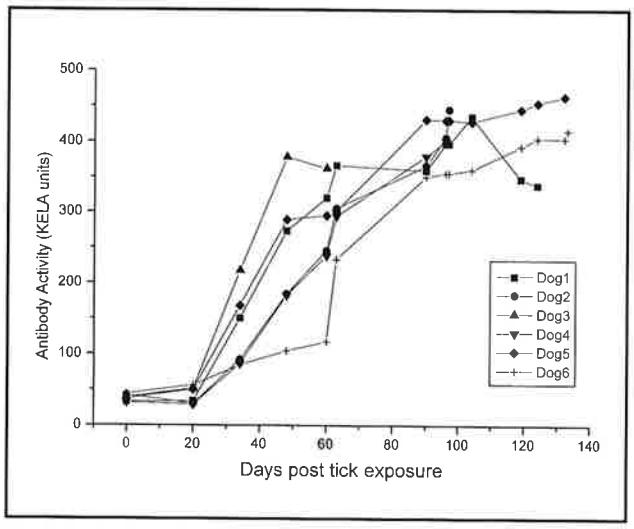

Fig 2. Antibody levels of dogs exposed at day 0 to B burgdorferi *infected adult ticks* (I scapularis) *as determined by KELA.*

Comparable data from human Lyme borreliosis are missing but we are speculating that a similar distribution of spirochetes can be found. Conflicting results are reported for spirochetemia in dogs,[22,28,32,49] rhesus monkeys,[50-52] and humans.[9,18,52]

The pattern of bacterial dissemination has a number of explanations. The disposition of the spirochete was related to the site of tick attachment (chest wall). Hence positive results were obtained with axillary and cervical lymph nodes, skeletal muscles, and fascias from the forelimbs but not from the hind limbs. Thus, in clinical patients, skin biopsy near the tick attachment site would be indicated. Positive results were recorded for the pericardium in 4 dogs while from the myocardium in only 1 and from the meninges in 2 dogs but never from brain parenchyma (cerebrum, cerebellum, or brain stem). Previous investigators have suggested that the Lyme disease agent may establish persistent infection within fibrous tissues.[54-56] Our results seem to be in accord with this hypothesis, as do our positive results from fascias, pericardium, and peritoneum. Further, it raises the question whether infection in other sites (skin, lymph nodes, skeletal muscle) is also due to the fibrous stroma of these organs. We have had very limited success in recovering *B burgdorferi* from body fluids. Isolation from joint fluid or CSF is rarely positive while joint capsules or meninges are sometimes positive.[22,33] This also suggests that the organism survives proficiently within connective tissue which may act as a sanctuary for *B burgdorferi,* protecting it from antibiotics and/or the immune system. In a previous study,[22] we reported that *B burgdorferi* can be isolated from the adrenal gland. We have not repeated that observation in this study. The agent appeared to persist only in the covering peritoneum, but not in the gland itself. Once we removed the peritoneum from adrendal glands, the culture became negative.

Recovery of *B burgdorferi* by conventional culture techniques and demonstration of *B burgdorferi* DNA by nested PCR gave closely comparable results in tissues that were positive (Tables 1 and 2). For example, of 36 synovia tested, 10 were positive for both culture and PCR. Borrelia were recovered from 12 of 36 lymph nodes while 14 of 36 were PCR positive. In the overall majority of cases, positive tissue samples were identified by both techniques. However, 13 of 55 positive specimens were culture positive/PCR negative and 10 of 55 specimens showed the reverse. The discrepancy was encountered in all positive sites (skin, synovial membranes, lymph nodes, fascias, etc) and so is not related to tissue factors. Separate pieces of tissue were taken for each test and a larger specimen was homogenized for culture compared with DNA extraction for PCR; this would favor a positive culture result. However, culture requires the presence of viable organisms while PCR simply detects DNA, which may be carried in fractions of the organism. A good correlation between culture and PCR was reported in some studies[20,25,26] while a higher incidence of PCR over culture results were reported in others.[18,31,41] One could question the sensitivity of the culture method or the specificity of the PCR. For example, we have found that the commercial BSK-H medium (Sigma Co., St Louis, Mo) was less suitable for isolation of *B burgdorferi* than our own BSK-II medium. Furthermore, the medium preparation is not standardized between laboratories. Conversely, false positive PCR results can sometimes be expected.

The frequency of clinical lameness in this study (three of six dogs) was similar to our earlier studies with 6-week-old puppies.[22] Lameness was seen in the right front limb in all three dogs, the limb closest to the site of tick exposure. We have seen this correlation in other tick-exposed, *B burgdorferi*-infected dogs (unpublished data). In recent reports, canine Lyme disease was induced by several cycles of tick exposure,[49,57] which resulted in a higher frequency of lameness. This latter protocol has merit, as it may resemble natural infection in the field where dogs (and other animals) could be infected by several waves of ticks.

The serological results (KELA), Western blots, and histopathologic results were in accord with our previous studies.[22] Persistent infection induces a plasma cell and lymphocyte-rich inflammation in joint capsules and is frequently seen as polyarthritis. Lymph nodes show marked plasma cell formation in medullary zones and germinal follicle formation is heightened.

Our studies of Lyme disease in the dog have been stimulated to facilitate control of this newly recognized

canine pathogen, and as a valuable model for human borreliosis. The ideal model for human disease will closely mimic the clinical, immunological, and pathological aspects of the infection in people.

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Journal of Spirochetal and Tick-borne Diseases

Dedicated to science and art in spirochetal and tick-borne diseases

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The *Journal of Spirochetal and Tick-borne Diseases* publishes quarterly reviews and original-work studies about any aspect of spirochetal and tick-borne diseases. The primary purpose is to broaden our understanding of spirochetal and tick-borne diseases. Special focus is given to Lyme borreliosis (also known as Lyme disease), as the most prevalent spirochetal and tick-borne disease. The clinical topics may involve all medical disciplines, nursing, and pharmacy, as well as the social, ethical, and biological features of spirochetal and tick-borne diseases.

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