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Animal Model

Chronic Lyme Borreliosis in the Laboratory Mouse

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C3H/HeJ mice were inoculated intraperitoneally with 10^7 uncloned* Borrelia burgdorferi *at 4 weeks of age and examined on days 30, 90, 180, and 360. Spirochetes were isolated from multiple tissues at all intervals. Joint and heart disease were present in all mice at 30 days and resolved after 90 days. At 180 and 360 days, some mice had mild recurrent joint and heart disease, and most had peripheral segmental periarteritis. The protein electrophoretic migration of 360-day isolates differed from the original inoculum. The experiment was repeated with C3H/HeN and BALB/cByJ mice inoculated intradermally with 10^4 cloned* B. burgdorferi *Characterization of infection and disease at 180 and 360 days were similar to those of the first experiment, but spirochetal proteins of isolates from both intervals displayed no protein variation in electrophoretic mobilities. Spirochetes isolated at 360 days were fully pathogenic in naive mice. Sera from infected mice showed an initial immunoglobulin M response, followed by a sustained immunoglobulin G response, involving IgG1, IgG2a, IgG2b and IgG3, with expanding reactivity against multiple antigens over time. These results indicate that immunocompetent mice sustain persistent infections and develop early acute joint and heart lesions that resolve and then recur intermittently. (Am J Pathol 1993, 143:959–972)

Lyme borreliosis is a multisystem disease caused by *Borrelia burgdorferi*, which is transmitted primarily through the bite of *Ixodes* ticks. The most common form of Lyme disease in humans is characterized by a wide variety of musculoskeletal, cardiac, and neurological symptoms, which tend to remit spontaneously but recur intermittently when not interrupted by therapy. The multiple manifestations are believed to be due to hematogenous dissemination of spirochetes, with resulting arthritis, synovitis, myositis, carditis, and other lesions.[1,2] A minority of patients also develop severe, unremitting joint disease, which may have genetic factors in its pathogenesis.[3] Untreated patients with Lyme disease usually have persistently elevated and rising antibody titers to *B. burgdorferi*, with expanding reactivity to multiple antigens and intermittent reappearance of IgM antibody to *B. burgdorferi*, suggesting continuous antigenic exposure.[1,2,4] When patients are treated with antibiotics, antibody reactivity wanes.[4] The chronic, intermittent course of disease and the sustained and expanding antibody response in Lyme disease patients suggest that untreated patients may be persistently infected with *B. burgdorferi*. A number of patients have been shown to be infected with *B. burgdorferi* months to years after initial exposure.[5–7] These observations suggest that *B. burgdorferi* may evade immune clearance and persist in some or all patients, whether or not disease is manifest.

An understanding of the mechanisms of Lyme disease and spirochetal persistence in the immunocompetent host can best be approached with a laboratory animal model of the disease. We have shown that a number of genotypes of laboratory mice are susceptible to infection with *B. burgdorferi* and develop polysynovitis, arthritis, carditis, and vasculitis.[8–12]

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Mice could be infected with the fewest spirochetes when inoculated intradermally.[9] Disease severity is significantly influenced by mouse genotype and age.[10] When mice are inoculated intradermally at 3 or more weeks of age, genetic differences in disease severity are apparent. For example, C3H mice develop consistently severe joint and heart disease, BALB mice develop mild joint but severe heart disease, and C57BL/6 mice develop very mild joint and heart lesions.[8,10,11] The genetic difference in arthritis severity is apparent even in C3H and BALB mice with severe combined immune deficiency (SCID). Congenic, immunocompetent mice without the SCID mutation develop arthritis and carditis that peak in severity by days 14 to 30, with regression of these lesions by 60 days, whereas SCID mice develop progressively severe joint disease and persistent heart disease through 60 days. Despite disease regression in immunocompetent mice, both SCID and immunocompetent mice remained infected through 60 days, based on culture from a number of tissues.[12]

The purpose of the present study was to examine the long-term course of *B. burgdorferi* infection in immunocompetent laboratory mice. Two experiments were performed. In the first experiment, C3H/HeJ mice were inoculated intraperitoneally with 10^7 uncloned *B. burgdorferi* and followed for up to 360 days after inoculation. Based on results of that experiment and because of the lipopolysaccharide (LPS) unresponsive nature of C3H/HeJ mice,[13] a second experiment was executed in which arthritis-susceptible (and LPS-responsive) C3H/HeN and arthritis-resistant BALB/cByJ mice were inoculated intradermally with a lower dose (10^4) of a cloned population of *B. burgdorferi*. These studies indicate that immunocompetent mice develop persistent *B. burgdorferi* infections with an intermittent disease course, similar to Lyme disease in humans.

Materials and Methods

Mice

Random sex, virus antibody-free C3H/HeJ (C3H-J), and BALB/cByJ (BALB) mice were purchased from The Jackson Laboratory, Bar Harbor, ME, and C3H/HeNCrlBR (C3H-N) mice were purchased from Charles River Laboratories, Portage, MI. Mice were shipped in filtered crates, maintained in isolator cages (Lab Products, Maywood, NJ), and provided food (Agway, Syracuse, NY) and water *ad libitum*. They were killed with carbon dioxide gas, followed by cardiac exsanguination.

Borrelia burgdorferi

The N40 strain of *B. burgdorferi* is a tick isolate that has undergone only 3 *in vitro* passages and has proven infectivity and pathogenicity in mice.[8–12] The N40 clone was obtained by triplicate *in vitro* passage of terminal dilutions. Infectivity and pathogenicity of the N40 clone was then verified in C3H-N mice. Spirochetes were grown in modified Barbour-Stoenner-Kelly (BSK II)[14] medium at 33 C without antibiotics. Blood (2 drops), ear punch (1.5 mm diameter), and urinary bladder were collected aseptically and cultured by placing tissue directly into 8-ml glass screw-top tubes containing 7.0 ml medium. Aseptically collected kidney, liver, brain, and spleen were homogenized in 4× volume of BSK II medium, then 0.5 ml homogenate was transferred to a tube containing 7 ml medium. Cultures were incubated for 2 weeks, then examined for spirochetes by darkfield microscopy. Inocula were grown to log-phase, quantified with a blood counting chamber, and diluted to the desired concentration with BSK II medium.

Polymerase Chain Reaction (PCR)

Borrelia burgdorferi-specific outer surface protein (osp) A DNA was detected in ear punch samples as previously described.[11,15] Primers osp A 149 (5′-TTA TGA AAA AAT ATT TAT TGG GAA T-3′) and osp A 319 (5′-CTT TAA GCT CAA GCT TGT CTA CTG T-3′) were used, and isopsoralen was included in all reactions and controls to prevent false positives due to amplicon carryover. Reactions were performed in a Perkin-Elmer-Cetus (Norwalk, CT) thermal cycler. Components were denatured at 94 C for 30 seconds, annealed at 55 C for 45 seconds, and extended at 72 C for 1 minute, for a total of 45 cycles. Negative (no target) controls were included at every fifth sample; all such controls were consistently PCR-negative. Amplification products were slot-blotted and probed, as described.[11,15] All amplifications were performed under conditions recommended for the prevention of false positives.

Histology

Tissues were immersion-fixed in 10% neutral buffered formalin, pH 7.2. Bone was demineralized in decalcifying solution (Baxter Health Care Corp., McGaw Park, IL). Tissues were embedded in paraffin, then processed and sectioned by standard technique. Tissues were stained with hematoxylin

and eosin or a modified Dieterle silver stain[11] for visualization of spirochetes.

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Pellets of *B. burgdorferi* cultures were washed in phosphate-buffered saline (PBS) containing 5 mmol/L $MgCl_2$, lysed in distilled water, analyzed for protein content, placed in incubation buffer (5% 0.2 Trizma base neutralized with H_3PO_4 [pH6.8], 1% SDS, 1% mercaptoethanol, 48% urea in distilled water) to give a final concentration of 0.85 mg protein/ml. Samples were boiled for 5 minutes, then processed by SDS-PAGE in a SE 600 vertical gel unit (Hoefer Scientific Instruments, San Francisco, CA) as described.[16] Each lane was loaded with 15 µl of protein solution. Gels were stained with Coomassie brilliant blue R-250.

Serology

Sera were tested for IgM and IgG reactivity to whole *B. burgdorferi* N40 by enzyme-linked immunosorbent assay (ELISA). Plates were coated with 0.125 ug intact *B. burgdorferi*/well and allowed to dry overnight at 37 C, blocked with 3% gelatin in PBS for 1 hour, then washed three times in PBS at 37 C. Serial twofold serum dilutions, beginning at 1:80 were added to wells and incubated for 1 hour. Plates were washed with PBS, treated with horseradish peroxidase-conjugated goat anti-mouse immunoglobulin M (IgM) or IgG (Tago Inc., Burlingame, CA) for 1 hour at 37 C, washed three times, followed by incubation with 3,3′,5,5′-tetramethylbenzidine and H_2O_2 (Kirkegaard and Perry Laboratories, Gaithersburg, MD). After 20 minutes, 100 ul 1 N HCl was added to each well to stop the reaction, and absorbance was determined at a wavelength of 450 nm.

Immunoblots were prepared by transfer of N40 proteins from a 15% acrylamide resolving gel and 3% stacking gel to nitrocellulose paper as described[16] with a Hoefer Transphor cell and power lid (Hoefer Scientific Instruments, San Francisco, CA). Molecular weight standards were loaded in separate, peripheral lanes, and N40 proteins were loaded in a continuous broad central lane. The gel was run for 5 hours at 30 mAmp, and proteins were transferred to nitrocellulose paper at 100V and 600 mAmp. Nitrocellulose paper was cut into strips, blocked overnight with 10% calf serum (Gibco Bethesda Research Laboratories, Gaithersburg, MD) in Tris-buffered saline (TBS), pH 6, rinsed with TBS, then incubated with 1:50 dilutions of test sera for 2 hours at room temperature on a shaker. They were subsequently washed three times in TBS, incubated for 2 hours with a 1:500 dilution of goat anti-mouse IgM, IgG (Kirkegaard and Perry Laboratories, Inc.), IgG1, IgG2a, IgG2b, or IgG3 (Southern Biotechnology Associates, Inc., Birmingham, AL), rinsed with TBS, stained with 5-bromo-4-chloro-3-indolylphosphate/nitroblue tetrazolium phosphatase substrate system (Kirkegaard and Perry Laboratories, Inc.) until color development, and then rinsed in distilled water to stop the reaction. All sera were tested on nitrocellulose strips prepared from the same protein transfer. Molecular weights of key *B. burgdorferi* proteins were further verified by reaction of nitrocellulose strips with a cocktail of monoclonal antibodies to Osp A, Osp B, and flagellin (kindly provided by Fred S. Kantor, Yale University School of Medicine).

Experimental Plan

In an initial experiment, C3H-J mice were inoculated intraperitoneally with 10^7 uncloned *B. burgdorferi* N40 and examined at 30, 90, 180, and 360 days after inoculation (Tables 1 and 2). On all but day 360, ear punch, spleen, and kidney were cultured and brain, heart, kidney, urinary bladder, and joints (bilateral shoulder, elbow, carpus, hip, knee, tibiotarsus) were examined for histopathology. On day 360, blood, liver, brain, and urinary bladder were also cultured, and lung, liver, eye, skin, intestine, pancreas, salivary glands, lymph nodes, and reproductive organs were also examined for histopathology. In lieu of culture, 360-day ear punches were tested by PCR for osp A DNA. Isolates cultured from a number of tissues at 360 days were compared by SDS-PAGE, and their infectivity and pathogenicity were examined by intradermal inoculation of naive C3H-N mice. During the course of this experiment, other studies found that intradermal inoculation was more efficient at establishing infection (lower median infectious dose) compared to intraperitoneal inoculation.[9]

Because the initial experiment demonstrated SDS-PAGE differences among *B. burgdorferi* isolates from 360-day mice compared to the original inoculum and because the experiment was based upon intraperitoneal inoculation (rather than the optimal intradermal route) of large numbers of uncloned spirochetes in C3H-J mice, which are

Table 1. *Isolation of* B. burgdorferi *from Different Organs of C3H-J Mice at Intervals after Intraperitoneal Inoculation with 10^7 Uncloned* B. burgdorferi *N40 or C3H-N and BALB Mice after Intradermal Inoculation with 10^4 Cloned* B. burgdorferi *N40*

Inoculum	Genotype	Interval (days)	Culture						
			Spleen	Ear	Kidney	Blood	Liver	Brain	Bladder
Uncloned N40	C3H-J	30	7/9*	2/2	1/10				
		90	5/9	5/5	2/9				
		180	8/10	2/2	3/10				
		360	6/10	(10/10)†	3/9	2/10	1/9	0/9	5/6
Cloned N40	C3H-N	180		1/1	0/4	1/7			4/4
		360		5/6	0/6	2/7		0/7	3/6
Cloned N40	BALB	180	1/1		0/7	0/7			5/6
		360		6/6	0/6	1/6		0/6	2/6

* Number positive/number cultured. Denominators vary due to deletion of contaminated samples.
† Day 360 ear samples were tested by PCR for osp A DNA.

Table 2. *Incidence of Active Disease at Intervals after Intraperitoneal Inoculation of C3H-J Mice with 10^7 Uncloned* B. burgdorferi *N40 or Intradermal Inoculation of C3H-N and BALB Mice with 10^4 Cloned* B. burgdorferi *N40*

Inoculum	Genotype	Interval (days)	Carditis	Arthritis
Uncloned N40	C3H-J	30	10/10*	10/10
		90	1/10	4/10
		180	0/10	5/10
		360	4/11	1/11
Cloned N40	C3H-N	180	0/4	0/4
		360	3/6	1/7
	BALB	180	0/6	6/7
		360	0/6	2/6

* Number of mice with active lesions/number of mice examined. Data include only active (acute) lesions and exclude chronic residual lesions.

LPS-unresponsive,[13] a second experiment was commenced. The course of infection in arthritis-susceptible C3H-N (LPS-responsive) and arthritis-resistant BALB mice inoculated intradermally with 10^4 cloned *B. burgdorferi* N40 was examined. Sera were collected from these mice on days 14, 30, 90, 180, and 360. On days 180 and 360, ear, blood, urinary bladder, kidney, and brain (360 days only) were cultured. Brain, lung, kidney, liver, urinary bladder, eyes, heart, joints, and skin were examined for histopathology. Sera were tested for IgM and IgG titers against *B. burgdorferi* by ELISA and immunoglobulin class/subclass (IgM, IgG, IgG1, IgG2a, IgG2b, IgG3) reactivity by immunoblot. Isolates from mice infected for 180 and 360 days were compared by SDS-PAGE and tested for infectivity and pathogenicity as in the first experiment.

Results

Infection and Disease in C3H-J Mice

Most if not all C3H-J mice remained infected for periods up to 1 year after intraperitoneal inoculation with 10^7 *B. burgdorferi*. Following an initial phase of acute joint and heart disease, lesions regressed, followed by recurrent mild active disease in a few mice at later intervals.

Based upon culture and PCR results, C3H-J mice remained persistently infected with *B. burgdorferi* through 360 days after intraperitoneal inoculation (Table 1). Ear punches were the most consistently positive site of isolation, but many samples were contaminated with dermal bacteria, despite efforts to disinfect the skin before collection. At 180 days, nine out of 10 mice and at 360 days, eight out of 10 mice had positive cultures from one or more tissues. Due to bacterial contamination of some samples, it could not be concluded that some mice cleared the infection. Therefore, PCR was utilized to test skin samples at the 360-day interval, showing that 10 out of 10 ear punches were positive.

All mice sampled at 30 days had active joint and heart disease, which declined in prevalence at subsequent intervals (Table 2). Mice had acute inflammation of joints, bursae, tendon sheaths, ligaments, tendons, and attachment sites as detailed previously.[11] Synovium was hypertrophied and hyperplastic, with exudation of neutrophilic leukocytes and fibrin into the lumina (Figure 1a). Periarticular connective tissue was edematous and infiltrated with neutrophilic leukocytes and macrophages. In some sites, areas of proliferating fibroblasts contained multinucleated cells (Figure 1b). Multiple joints of each of all four limbs, including shoulder, elbow, carpus, metacarpus, phalanges, hip, knee, tarsus, and metatarsus, were affected in all mice. Bone adjacent to areas of intense inflammation had periosteal proliferation and osteoclasis (Figure 1c), and ligaments and tendons had fibroblastic proliferation, especially at osseous attachment sites. All 10 mice at this interval also had carditis, with acute inflammation of the root of the aorta above the aortic

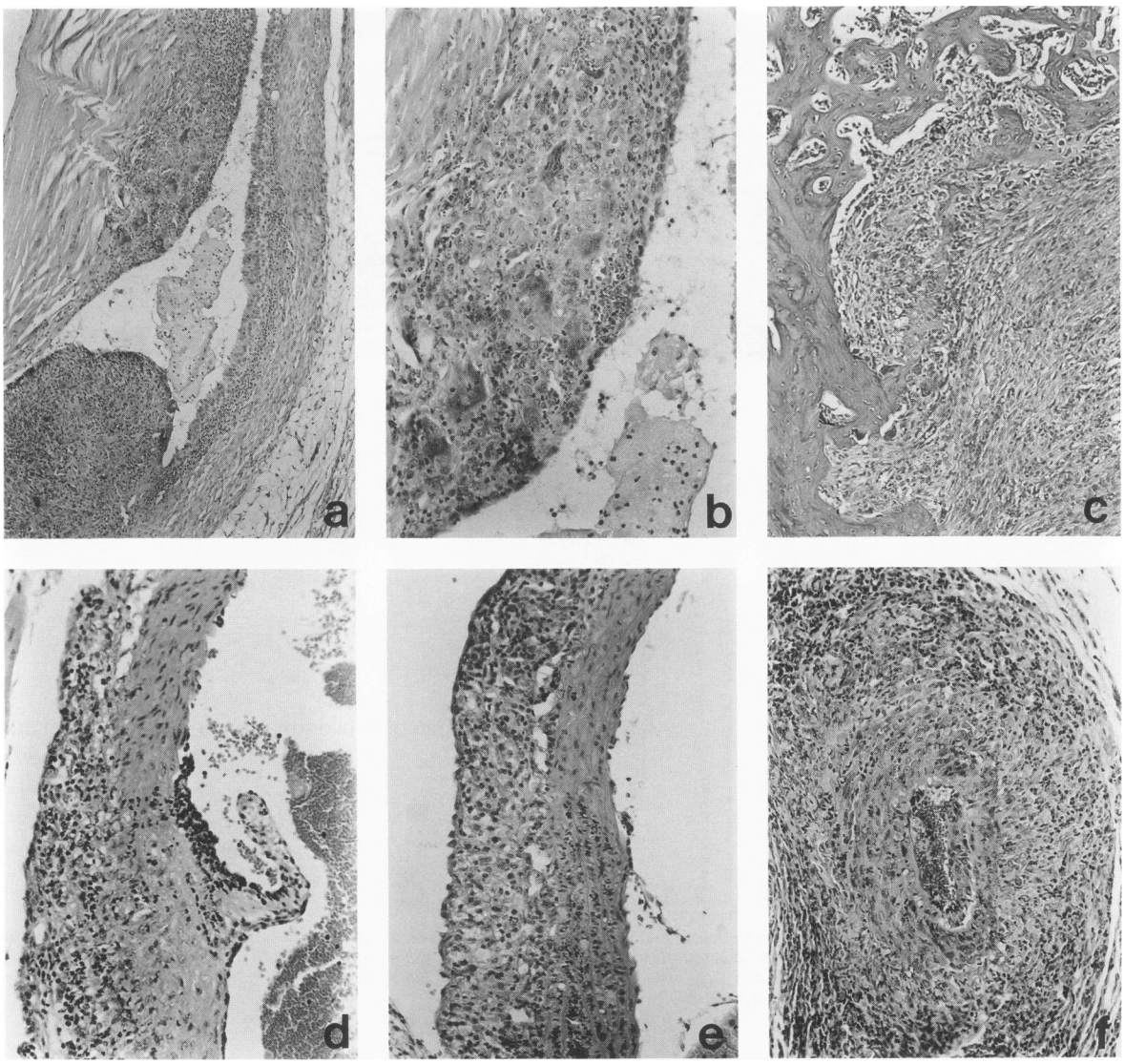

Figure 1. *Tissues of C3H-J mice at 30 days after* Borrelia burgdorferi *inoculation.* **a:** *Periarticular edema and acute fibrinopurulent synovitis, tibiotarsal region.* **b:** *Synovium of tendon from* **a**, *depicting multinucleate cells and fibroplasia.* **c:** *Fibroplasia and osteoclasis adjacent to synovial inflammation.* **d:** *Margination of leukocytes on the endothelium and transmural inflammation near aortic valve of heart.* **e:** *Transmural inflammation with thickening of the ascending aortic adventitia.* **f:** *Acute transmural femoral arteritis near knee.* **a**, **d**, **e**, **f**, *54×; b, c, 136×.*

valve, aortic adventitia, connective tissue at the base of the heart, epicardium, and endocardium (Figure 1, d and e), as described in detail previously.[8,11] Three of the 10 mice examined also had segmental inflammation of the femoral artery, plantar, and dorsal branches of the saphenous artery and/or the cranial tibial artery (Figure 1f). These vessels were not represented in sections from all mice, so the true prevalence of this lesion could not be assessed. Lesions in other tissues were absent, except for perivascular lymphocytic infiltrates in the submucosa of the urinary bladder in six of the mice.

At 90 days, less than half of mice had active arthritis, which was present in only one or two joints among all limbs examined of each mouse. The majority of affected joints were phalanges. There was evidence of disease resolution, with residual synovial fibrosis and exudation of macrophages into the synovial lumina and infiltration of the periarticular connective tissue with sparse populations of lymphocytes (Figure 2a). Many joints that were commonly affected at day 30 had no active disease, but revealed residual capsular fibrosis (Figure 2b). Six of the mice had segmental infiltration of arterial adventitia with lymphocytes, and in some vessels, acute focal endarteritis in these areas (Figure 2c). One mouse had acute arteritis above the aortic valve, whereas the other nine had dense bands of

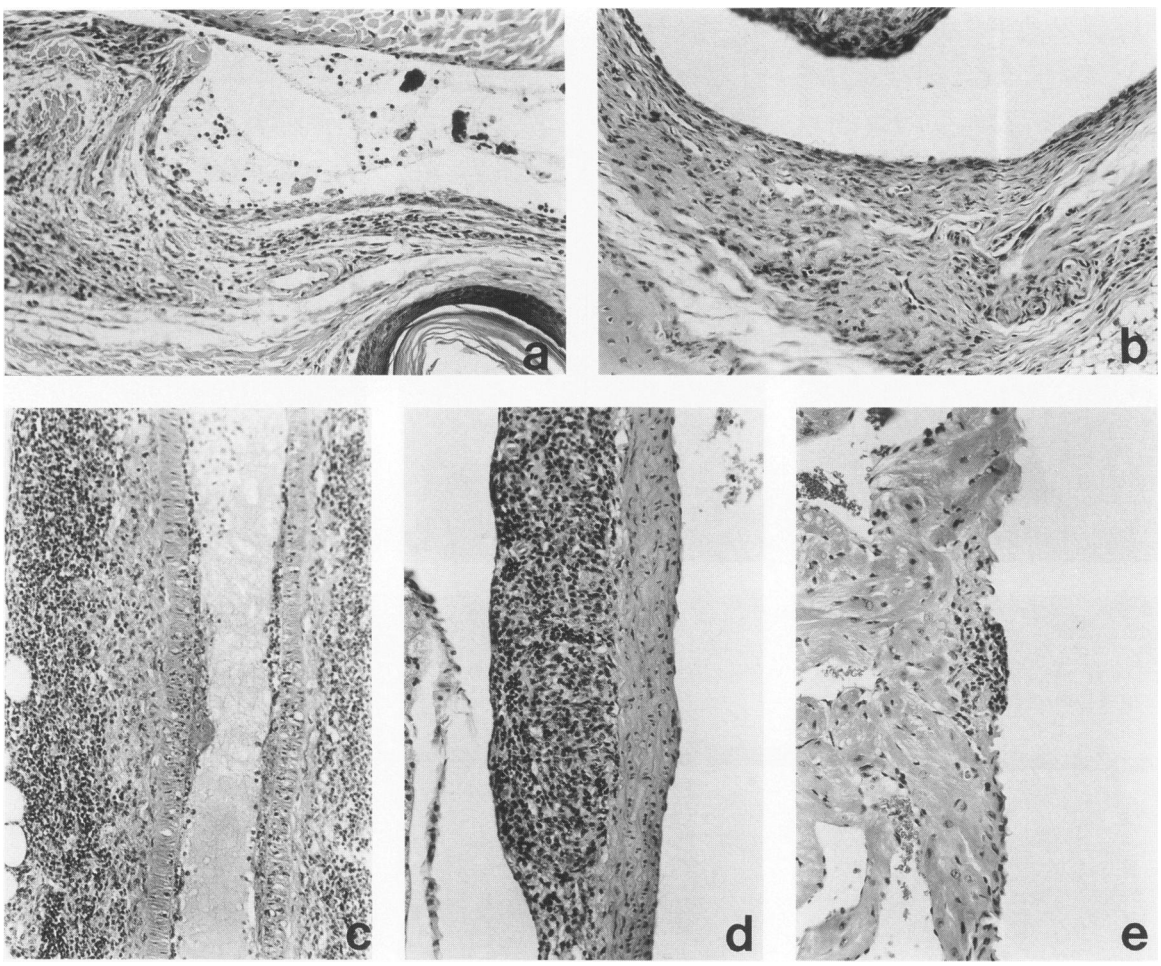

Figure 2. *Tissues of C3H-J mice at 90 days after* Borrelia burgdorferi *inoculation.* **a:** *Resolving tibiotarsal synovitis, with periarticular leukocyte infiltration and diminished luminal exudation.* **b:** *Resolved tibiotarsal synovitis with residual fibrosis.* **c:** *Segmental saphenous arteritis, with intense infiltration of the adventitia with lymphocytes and focal acute endarteritis.* **d:** *Segmental infiltration of the ascending aortic adventitia with lymphocytes.* **e:** *Focal infiltration of the atrial epicardium with lymphocytes.* **a** *to* **e**, *136×.*

lymphocytes and plasma cells in the adventitia of the aortic wall without acute inflammation above the aortic valve (Figure 2d). There were also small focal infiltrates of lymphocytes in the atrial epicardium (Figure 2e). All but one of the mice had perivascular lymphocytic infiltrates in the urinary bladder, and most also had lymphocytic infiltration of renal interlobular arterial adventitia.

At 180 days, five of 10 mice had active inflammation with mild fibrinopurulent exudation of individual joints (Figure 3a). The other mice had no evidence of joint disease, but eight had segmental periarteritis. Most of the mice also had perivascular lymphocytic infiltrates in urinary bladder and kidney. None of the mice had active cardiac lesions, but all had lymphoplasmacytic infiltration of the aortic adventitia at the heart base. One of the 11 mice examined at 360 days had active arthritis, and several had residual lesions, including focal ossification of tendons and ligaments, especially the Achille's tendon and patellar ligament. Four of the mice had active inflammation of the aortic wall above the aortic valve (Figure 3b), and all had lymphoplasmacytic infiltration in the aortic adventitia (Figure 3c). Seven of the mice had segmental lymphocytic infiltration of peripheral arterial adventitia. Most mice had perivascular lymphocyte cuffing in the urinary bladder, kidney, and lungs.

Infection and Disease in C3H-N and BALB Mice

The course of infection following intradermal inoculation of C3H-N and BALB mice with 10^4 cloned *B. burgdorferi* was similar to that of C3H-J mice inoculated intraperitoneally with 10^7 uncloned *B. burgdorferi* (Tables 1 and 2). Because course of infec-

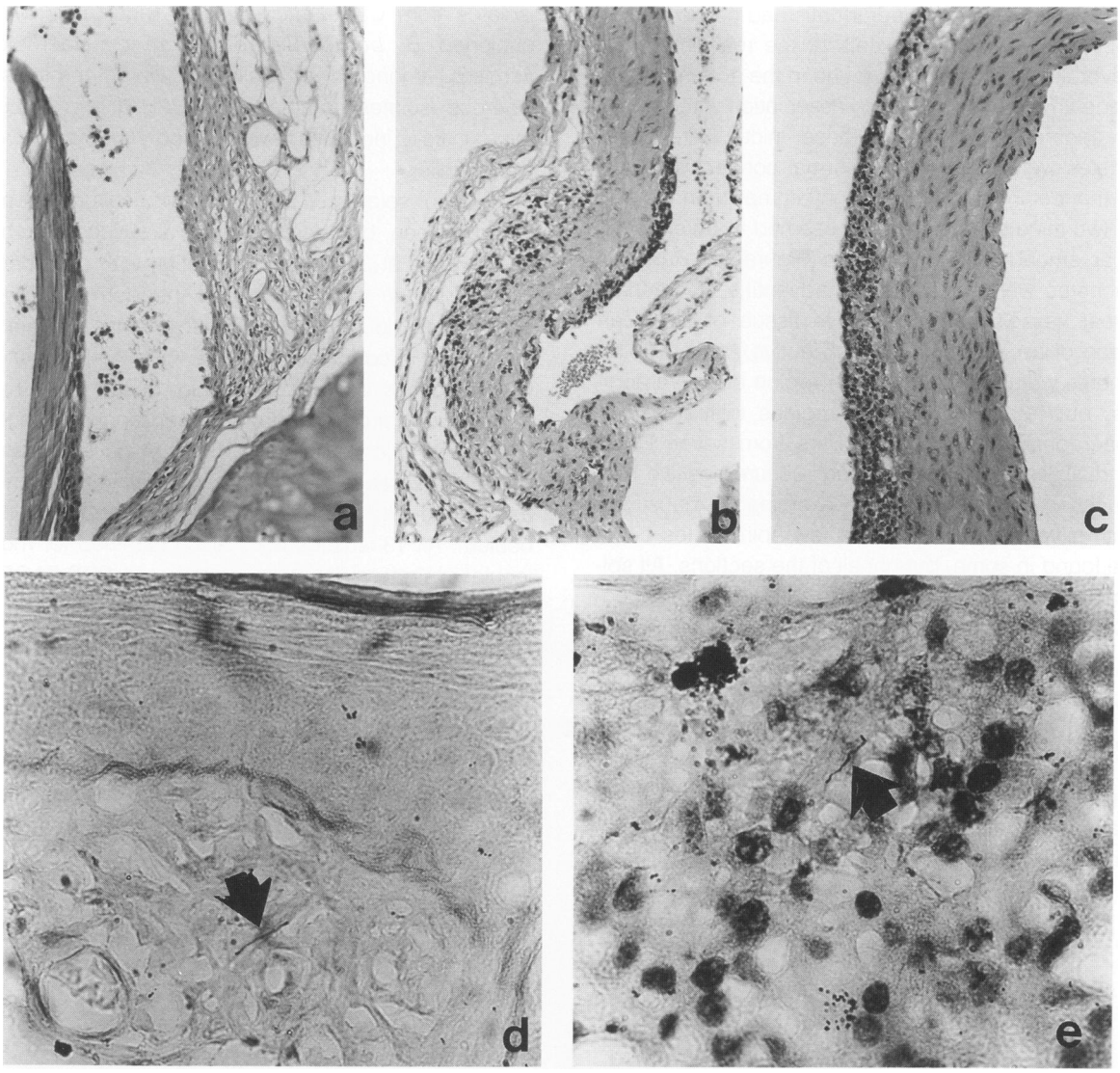

Figure 3. *Tissues of C3H mice at 180 or 360 days after* Borrelia burgdorferi *inoculation.* **a:** *Active tibiotarsal synovitis at 180 days.* **b:** *Active aortitis above the aortic valve at 360 days.* **c:** *Segmental infiltration of the ascending aortic adventitia with lymphocytes at 360 days.* **d:** *Spirochete in dermis of rear foot at 360 days. (Dieterle stain).* **e:** *Spirochete in connective tissue of heart base at 360 days (Dieterle stain).* **a**, *54×;* **b**, **c**, *136×;* **d**, **e**, *790×.*

tion and disease has already been studied through 60 days in these genotypes of mice following intradermal inoculation with 10^4 *B. burgdorferi* N40,[10–12] tissues from mice were cultured and examined for disease on days 180 and 360 only. Spirochetes were isolated from one or more tissues of most mice at these intervals, regardless of genotype (Table 1). At 180 days, five out of six BALB and four out of seven C3H-N mice had positive cultures from one or more tissues, and at 360 days, six of six BALB and six of six C3H-N mice were culture-positive. As with C3H-J mice, it could not be concluded that some mice cleared infection, as some samples were contaminated. Joint and heart lesions resembled those seen in C3H-J mice, with evidence of arthritis resolution in many joints, active disease in others, and chronic lymphoplasmacytic infiltrates around the root of the aorta. Most BALB mice had active but mild inflammation of one or more joints at 180 days, whereas C3H-N mice did not, and two of six BALB and one of seven C3H-N mice had active joint disease at 360 days (Table 2).

Visualization of Spirochetes in Tissue Sections

Selected sections from 360-day C3H-J mice were Dieterle-stained to reveal presence of spirochetes.

Three mice with resolved arthritis had no visible spirochetes in or around joints of the rear limbs, but several spirochetes were found in the adventitia and beneath the endothelium in association with regions of segmental arteritis in these mice. Rare spirochetes were found in the dermal connective tissue of the feet in the absence of inflammation in the skin of two mice examined. They seemed to be extracellular, among collagen bundles (Figure 3d). Rare spirochetes were visible in the adventitia of coronary great vessels and connective tissue of the heart base of two of two mice with active aortitis. Spirochetes were not visible in the region of active aortitis, but rather in adjacent regions infiltrated with macrophages (Figure 3e). Ears from seven BALB and C3H-N mice that had culture-positive ear punches at 360 days were examined exhaustively with silver stain, and only a few spirochetes could be found in some, but not all of the sections. All spirochetes were extracellular in the dermal connective tissue.

Infectivity and SDS-PAGE Profiles of B. *burgdorferi Isolates*

Nearly all *B. burgdorferi* isolated from mice at 360 days were infectious and pathogenic when inoculated into naive C3H-N mice. SDS-PAGE profiles of isolates from C3H-J mice, initially inoculated with uncloned *B. burgdorferi*, displayed considerable variation among lower molecular weight proteins, whereas isolates obtained from C3H-N and BALB mice, initially inoculated with cloned *B. burgdorferi*, were similar.

Thirteen isolates of *B. burgdorferi*, cultured from blood, spleen, bladder, or kidney of seven different C3H-J mice at 360 days of infection with uncloned *B. burgdorferi* were inoculated intradermally (10^4 spirochetes/mouse) into two to four naive, 3-week-old C3H-N mice for each isolate. All but one isolate was infectious and/or pathogenic, based upon reisolation of spirochetes from spleen and presence of disease in joints and/or heart at 14 days after inoculation. Disease severity was relatively the same as that induced with the original, uncloned N40 inoculum. All 13 isolates were also examined for molecular weight variation of proteins on SDS-PAGE. Several seemed to differ from the original, uncloned N40 inoculum (Figure 4), including variation among isolates from different organs of the same mouse. The major variation occurred in lower molecular weight bands (20 to 30 kd), although subtle differences were also apparent among other bands.

Ten *B. burgdorferi* isolates from bladder, ear, and blood of six C3H-N mice and nine isolates from kidney, bladder, ear, and blood of six BALB mice at

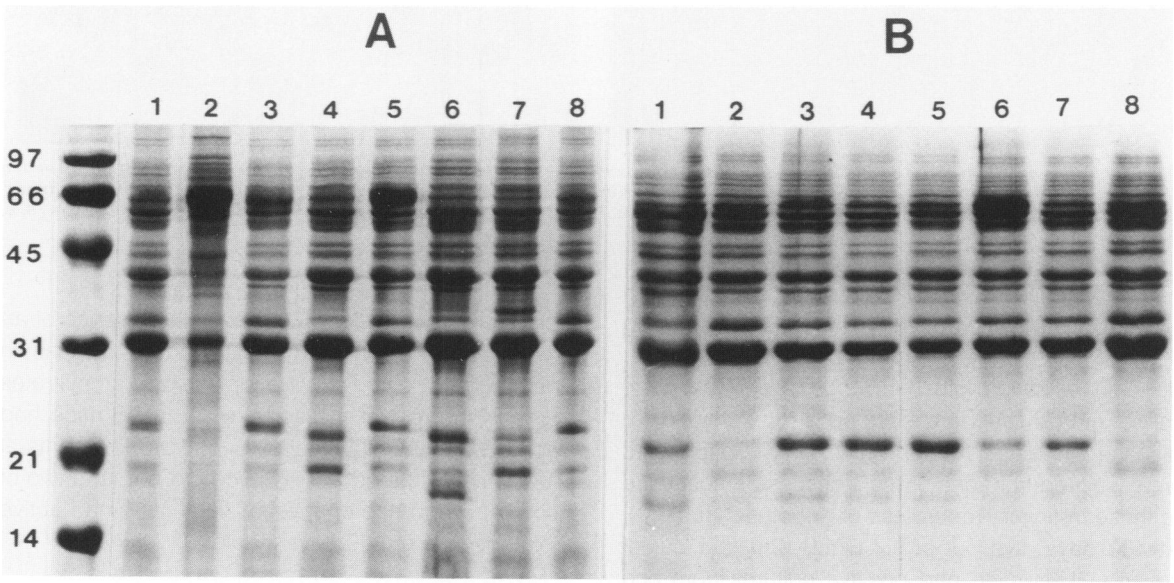

Figure 4. *SDS-PAGE profiles of selected* Borrelia burgdorferi *isolates at 360 days after inoculation. Molecular weight markers (in kilodaltons) are on the left.* **A, lanes 1** *and* **8:** *uncloned* Borrelia burgdorferi: **lanes 2** *to* **7:** Borrelia burgdorferi *isolated from 123 spleen, 130 spleen, 130 bladder, 131 bladder, 132 kidney, 132 bladder.* **B, lane 1:** *uncloned* Borrelia burgdorferi, **lanes 2** *and* **8:** *cloned* B. burgdorferi; **lanes 3** *to* **7** Borrelia burgdorferi *isolated from 747 kidney, 749 ear, 752 blood, 752 bladder, and 755 bladder. Note variation in lower molecular weight proteins among isolates from mice inoculated with uncloned* Borrelia burgdorferi (**A**) *compared to uniformity among isolates from mice inoculated with cloned* Borrelia burgdorferi (**B**).

360 days of infection with cloned *B. burgdorferi* were tested for infectivity and pathogenicity by intradermal inoculation of 10^4 spirochetes into naive C3H-N mice. Based on culture of spleen and bladder and disease in joints and/or heart at 14 days after inoculation, all isolates were both infectious and pathogenic, with no relative difference compared to the cloned, N40 inoculum. All isolates from both C3H-N and BALB mice at 180 and 360 days were examined on SDS-PAGE. In contrast to the initial experiment in C3H-J mice inoculated with uncloned *B. burgdorferi*, no variation in the molecular weights of proteins was observed among isolates from C3H-N or BALB mice inoculated with cloned *B. burgdorferi* (Figure 4). Proteins in the 20 kd range were identical in weight, but seemed to have variable density of bands.

Serological Response

The serological responses of mice killed at various time intervals after intradermal inoculation with 10^4 cloned *B. burgdorferi* were tested by ELISA and Western blots. Serial samples from individual mice were not collected. Sera from C3H-N and BALB mice were tested for antibody to *B. burgdorferi* by ELISA at intervals after intradermal inoculation (Table 3). IgM was detected by ELISA in most mice at the 14- and 30-day intervals, but not thereafter, except for a single C3H-N mouse at 180 days. This single IgM-positive mouse was notably the only spirochetemic animal among 14 mice sampled at 180 days. IgG ELISA titers were generally higher in C3H-N mice compared to BALB mice through 180 days. IgG ELISA titers rose in both genotypes through 180 days, then dropped significantly on day 360.

Sera from two or three C3H-N mice from each of days 14, 90, 180, and 360 (Figure 5) and BALB mice from days 14, 180, and 360 were tested for IgM, IgG, and IgG subclasses 1, 2a, 2b, and 3 by immunoblots against cloned *B. burgdorferi* N40 proteins (Table 4). IgM reactivity to 20-, 39-, and 41-kd proteins was found on day 14 in both genotypes and persisted as weak reactivity to these same proteins, without expansion of reactivity to other proteins, at later intervals. On day 14, C3H-N IgG reacted to 20- and 39-kd bands and BALB IgG reacted to the same bands, as well as to a 41-kd band. IgG1, 2a, 2b, and 3 reactivity was identical to IgG reactivity of each genotype, except BALB IgG2b, which did not react against the 41-kd protein. On day 90, C3H-N IgG reacted to 15-, 20-, 39-, 41-, and various higher (>45) kd molecular weight bands. IgG subclasses all reacted to the same bands, except reactivity to 31-kd protein was also detected with IgG1 and IgG2a. On day 360, both C3H-N and BALB IgG reacted to 15-, 20-, 31-, 34-, 39-, 41-, and higher kd molecular weight bands, but IgG subtypes IgG2b (BALB) and IgG3 (C3H-N and BALB) did not react to all bands. Thus, both complement-fixing and noncomplement-fixing IgG subclasses were represented in the murine serological response to *B. burgdorferi*.

Because we have previously noted reactivity to 31-kd (Osp A) protein before day 30 in experimentally infected mice inoculated intradermally with 10^4 *B. burgdorferi* N40[11] but did not find such reactivity in the present study, we immunoblotted sera from additional C3H-N mice at 21 days after intradermal infection (verified by subsequent culture of spleen and presence of joint and heart disease) with 10^1, 10^2 or 10^4 N40 *B. burgdorferi* to determine if seroconversion to Osp A is a dose-related phenome-

Table 3. *IgM and IgG Antibody Response to* B. burgdorferi *of C3H-N and BALB Mice at Intervals after Intradermal Inoculation with 10^4 Cloned* B. burgdorferi

Interval (days)	Genotype	Number positive/number tested, geometric mean titer (range)	
		IgM	IgG
14	C3H-N	3/3 640*	3/3 1,016 (640–2,560)
	BALB	4/4 320	4/4 905 (640–1,280)
30	C3H-N	4/4 269 (160–320)	4/4 24,355 (20,480–40,960)
	BALB	4/5 269 (160–320)	5/5 10,240
90	C3H-N	0/5	5/5 46,993 (20,480–163,840)
	BALB	0/5	5/5 40,960
180	C3H-N	1/7 160	7/7 973,861 (655,360–2,621,440)
	BALB	0/7	7/7 723,576 (327,680–2,621,440)
360	C3H-N	0/7	7/7 180,894 (163,840–327,680)
	BALB	0/6	6/6 216,188 (163,840–327,680)

* Number positive (≥ 80)/number tested. Titers expressed as reciprocal geometric means of positive sera (range of positive titers).

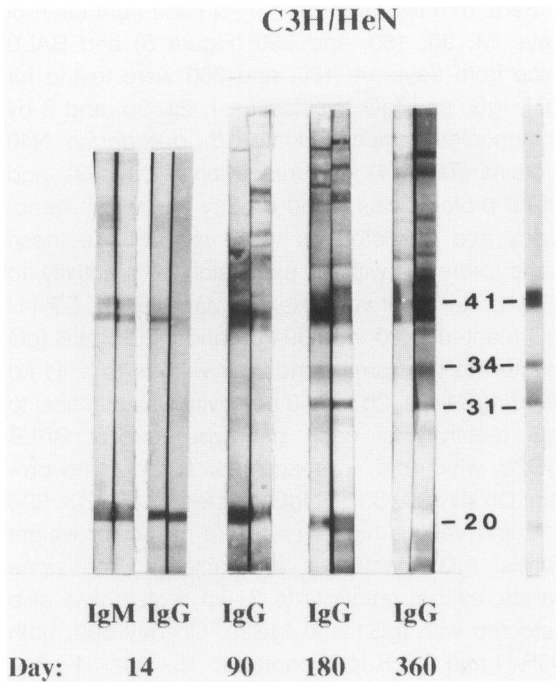

Figure 5. Borrelia burgdorferi *immunoblots of selected C3H-N mouse sera at 14, 90, 180, and 360 days after inoculation, depicting sequential expansion of antibody reactivity to spirochetal antigens. The strip on the right is labeled with mouse monoclonal antibody to Osp A (31 kd), Osp B (34 kd), and flagellin (41 kd).*

non. Three of four mice infected with 10^4 spirochetes reacted weakly to Osp A, but none of three mice and none of two mice seroconverted to Osp A at the 10^2 or 10^1 infecting doses, respectively. All infected mice seroconverted to 39-kd and 20-kd proteins.

Discussion

These results indicate that C3H-J, C3H-N, and BALB laboratory mice sustain persistent infections following inoculation with *B. burgdorferi*, with an initial bout of acute disease followed by disease remission in the early weeks of infection. In addition, it is apparent that mice develop recurrent active disease in joints and hearts, but disease that is milder than the early phase of infection. Active lesions seen at the 180- and 360-day intervals were not chronic in nature, but rather had features similar to the early phase of infection, suggesting recurrence rather than persistence of lesions. Our previous studies have indicated that regression of arthritis and carditis coincides with clearance of spirochetes from these target sites.[8,11] Periodically, spirochetes may cause disease exacerbation following events that allow recurrent spirochetemia and reinvasion of target tissues. We have found that nearly all mice are demonstrably spirochetemic in the early phase of infection, but later clear spirochetes from peripheral blood at 21 to 30 days, a time coinciding with onset of arthritis regression.[11,12] In the present study, a fraction of mice at 6 and 12 months were spirochetemic, and it is tempting to speculate that they were in the early phase of disease recurrence. The milder disease and fewer joints affected in the late stage of infection suggest that specific immunity attenuates the ability of reinvading spirochetes to proliferate and induce severe disease. Alternatively, the milder recurrent disease seen in mice in the late stage of infection may also be a reflection of age-related resistance to disease. Adult (12-week) C3H mice inoculated intradermally with *B. burgdorferi*, developed less severe disease compared to mice inoculated at 3 to 4 weeks of age.[10] Our studies in SCID and congenic immunocompetent mice indicate that synovium is a preferential site of spirochete proliferation, but that the immune system abrogates this process, resulting in elimination of spirochetes and arthritis regression in the immunocompetent host.[12] This pattern of persistent infection, acute disease, disease remission, and intermittent bouts of exacerbation is typical of untreated human Lyme disease.[1,2] These studies also show provocative evidence of *B. burgdorferi* persistence in the immunocompetent host, which has been demonstrated in a few human patients[5–7] as well as in experimentally infected rats and hamsters.[17–19]

The phenomenon of arthritis and carditis regression that occurs after the early phase of infection does not seem to be due to a change in the biological behavior of the spirochetes. Spirochetes isolated from a number of tissues and mice at 12 months after inoculation retain their ability to infect and produce disease in naive mice, despite the fact that they were isolated from hosts with minimal or no disease. It could be argued that such biological behavior may revert in the process of culturing these organisms *in vitro*, but such a process usually diminishes infectivity[20–22] rather than accentuates it.

In the first experiment, we were intrigued by the overt SDS-PAGE molecular weight differences among spirochetes isolated from different mice and among spirochetes isolated from different organs of the same mouse. This prompted the second experiment, in which mice were inoculated with a cloned strain of *B. burgdorferi*. Spirochetes isolated from mice infected with cloned *B. burgdorferi* did not display SDS-PAGE variation. Thus, the variation noted in the first experiment was probably due to clonal selection of different subpopulations of spirochetes within the uncloned, high-dose inoculum. However,

Table 4. *Summary of C3H-N and BALB IgG and IgG Subclass Immunoblot Reactivity to Major* B. burgdorferi *Proteins at Intervals after Intradermal Inoculation*

Interval (days)	Genotype	Isotype	Protein molecular weight						
			>45	41	39	34	31	20	15
14	C3H-N	IgG,1,2a,2b,3	–	–	+	–	–	+	–
	BALB	IgG,1,2a,2b,3	–	+(2b-)	+	–	–	+	–
90	C3H-N	IgG	+*	+	+	–	–	+	+
		IgG1	+	+/–	+	–	+/–	+	+
		IgG2a	+	+	+	–	+	+	+
		IgG2b	+	+/–	+	–	–	+	+
		IgG3	+	+	+	–	+/–	+	+
180	C3H-N	IgG	+	+	+	+	+	+	+
		IgG1	+	+	+	–	+/–	+	+
		IgG2a	+	+	+	–	–	+	+
		IgG2b	+	+	+	–	+	+	+
		IgG3	+	+	+	–	–	+/–	–
	BALB	IgG	+	+	+	+	+	+	+
		IgG1	–	+	–	–	–	+	–
		IgG2a	+	+	+	–	–	+	–
		IgG2b	+	+	+	–	+/–	+	–
		IgG3	+	+	+	–	–	+	+
360	C3H-N	IgG	+	+	+	+	+	+	+
		IgG1	+	+	+	+	+	+	+
		IgG2a	+	+	+	+	+	+	+
		IgG2b	+	+	+	+	+	+	+
		IgG3	–	+	+	–	–	+	+
	BALB	IgG	+	+	+	+	+	+	+
		IgG1	+	+	+	+	+	+	+
		IgG2a	+	+	+	+	+	+	+
		IgG2b	+	+	+	+	–	+	+
		IgG3	+	+	+	+	–	+	+

* Positive (+), negative (–) or weak (+/–) reactivity of 2 to 3 mouse sera at each interval.

uniformity of SDS-PAGE profiles among isolates in the second experiment does not rule out more subtle variation at the structural or antigenic level. There is mounting evidence that osp A and B may display such variation. Recombination between homologous genes encoding osp A and B, with deletions and chimeric gene fusions have been documented, resulting in variation of osp epitope expression.[23] *In vitro* exposure of *B. burgdorferi* to antibody to osp A and B has been shown to select for the evolution of spirochetes that lack Osp expression, have osp gene mutations or have lost the osp genes entirely.[24] Within our own uncloned *B. burgdorferi* stock, subpopulations of spirochetes with a stop codon in the Osp B gene, resulting in truncated Osp B lacking the protective epitope, were discovered when mice failed to be protected when vaccinated with full-length Osp B.[25]

Persistent infection seems to involve very small numbers of spirochetes. During the acute phase of infection, we have previously shown that spirochetes can be readily visualized in multiple tissues, especially in areas of joint and heart inflammation. They are also relatively plentiful in skin, but their numbers are significantly reduced if not entirely eliminated from hearts and joints during the regression phase of disease.[8,11] Very few spirochetes could be visualized in tissues of chronically infected mice in the present study, but they could be readily isolated or detected by PCR, especially in ear punch samples, in almost all mice at 360 days after initial infection. We have successfully utilized immunoperoxidase and *in situ* DNA hybridization for detection of spirochetes in tissue sections, but these methods have not been as sensitive as silver stain for identification of spirochetes,[8,11] possibly because of their lesser ability to penetrate tissues beyond the plane of section. The site of spirochete persistence remains a paradox, but this study strongly suggests that skin may be a preferred site and that the multiple organisms visualized in this organ were all located extracellularly. Furthermore, we have shown that the skin plays a facilitating role in establishing infection, as the intradermal median infectious dose is significantly lower than the infectious dose of organisms given by direct intraperitoneal injection.[9]

Immunoblot analysis of sera from infected mice revealed a conspicuous early reactivity of both IgM and IgG against 39-kd and approximately 20-kd proteins. Others have noted that white-footed and laboratory mice infected by syringe inoculation with low-passage infectious spirochetes or by tick inoculation, but not with dead or noninfectious spiro-

chetes, seroconvert to the 39-kd protein at an early stage of infection.[22,26] This *B. burgdorferi*-specific protein is reported to be recognized by human Lyme disease patients, and reactivity to the 39-kd protein may be misinterpreted as reactivity to the closely migrating 41-kd flagellin.[27] Flagellin did not elicit an IgG response in mice until the 90-day interval and beyond. Mice also responded early in infection and strongly to an approximately 20-kd protein. Proteins in this molecular weight range have been shown to elicit intense reactivity with human Lyme disease sera.[28] As seen in human patients with Lyme disease, seroconversion to 31-kd Osp A and 34-kd Osp B did not occur in our mice until late in the course of infection. This is in contrast to previous reports from our own and other laboratories of an early Osp A and B recognition in mice.[11,29,30] This apparent disparity is likely due to inoculum dose. Sera from mice with verified infection at 21 days following intradermal inoculation of 10^4, 10^2, or 10^1 spirochetes had weak Osp A and B reactivity only at the 10^4 dose, but none at the lower infectious doses. We have also demonstrated that mice infected by tick feeding do not seroconvert to Osp A or B within 4 weeks after tick exposure (Fikrig E, Bockenstedt L, Barthold SW, Chen M, Tao H, Ali-Salaam P, Flavell RA: Naturally acquired human immunity to Lyme borreliosis. 1993, submitted). Thus, the finding of late seroconversion to Osp A and B in the present study correlates very well with natural infections in humans.[1,4] It is interesting that Osp A and B, which are expressed *in vitro* on the outer surface of the organism and represent a major fraction of the total protein in *B. burgdorferi*, go unrecognized for so long in the course of persistent infection.

The present study validates the laboratory mouse as a model of the common form of Lyme borreliosis in humans. The close parallels in clinical course and expanding immune response between mouse and human Lyme borreliosis strongly suggest the possibility that a high percentage of humans, like mice, may be persistently infected with *B. burgdorferi* despite apparent disease resolution. This has been demonstrated in a limited number of human patients, and should be further explored. The skin is a likely site for examining this phenomenon.

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