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Research Articles

Spinal cord involvement in the nonhuman primate model of Lyme disease

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Lyme borreliosis is a multisystemic disease caused by infection with various genospecies of the spirochete *Borrelia burgdorferi*. The organs most often affected are the skin, joints, the heart, and the central and peripheral nervous systems. Multiple neurological complications can occur, including aseptic meningitis, encephalopathy, facial nerve palsy, radiculitis, myelitis, and peripheral neuropathy. To investigate spinal cord involvement in the nonhuman primate (NHP) model of Lyme borreliosis, we inoculated 25 adult *Macaca mulatta* with *B. burgdorferi sensu strictu* strains N40 by needle ($N=9$) or by tick ($N=4$) or 297 by needle ($N=2$), or with *B. burgdorferi* genospecies *garinii* strains Pbi ($N=4$), 793 ($N=2$), or Pli ($N=4$) by needle. Immunosuppression either transiently (TISP) or permanently (IS) was used to facilitate establishment of infection. Tissues and fluids were collected at necropsy 7–24 weeks later. Hematoxylin and eosin staining was used to study inflammation, and immunohistochemistry and digital image analysis to measure inflammation and localize spirochetes. The spirochetal load and C1q expression were measured by TaqMan RT-PCR. The results showed meningoradiculitis developed in only one of the 25 NHP's examined, TISP NHP 321 inoculated with *B. garinii* strain Pbi. Inflammation was localized to nerve roots, dorsal root ganglia, and leptomeninges but rarely to the spinal cord parenchyma itself. T cells and plasma cells were the predominant inflammatory cells. Significantly increased amounts of IgG, IgM, and C1q were found in inflamed spinal cord. Taqman RT-PCR found spirochetes in the spinal cord only in IS-NHP's, mostly in nerve roots and ganglia rather than in the cord parenchyma. C1q mRNA expression was significantly increased in inflamed spinal cord. This is the first comprehensive study of spinal cord involvement in Lyme borreliosis.
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Lyme borreliosis is a multisystemic infection caused by tick-borne spirochetes of the *Borrelia burgdorferi* group.[1] It begins at the site of a tick-bite with a characteristic skin lesion, erythema migrans. From the skin, the spirochetes can disseminate to involve multiple organs, with a preference for the musculoskeletal system, the nervous system, and the heart.[1] Multiple neurological complications can occur, notably facial nerve palsy, meningoradiculoneuritis, and aseptic meningitis.[2]

Much of what we know today about spinal cord involvement in Lyme borreliosis is from clinical rather than pathological or experimental studies. Preferential involvement of the spinal roots and meninges was noticed early.[3,4] In contrast, myelitis occurs much less frequently. The rare opportunities when human Lyme neuroborreliosis has been examined at autopsy had shown perivascular inflammation of lymphocytes and plasma cells in the meninges, spinal cord, and nerve roots.[5] Lyme meningoradiculitis appears to be more prominent in European patients infected with *B. garinii* strains. However, all three genospecies of *B. burgdorferi* pathogenic to humans (*B. burgdorferi sensu stricto*, *B. afzlii*, and *B. garinii*) have been isolated from patients with Lyme meningoradiculitis.[6]

Our understanding of the pathogenesis of Lyme meningoradiculitis has been limited by a paucity of animal models featuring Lyme neuroborreliosis. Unlike relapsing fever borrelias,[7] mice and other small rodents infected with Lyme disease borrelias rarely develop neuroborreliosis. For the last several years, we have been studying the pathogenesis of Lyme neuroborreliosis in nonhuman primates

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(NHP).[8–13] Previous studies showed that the localization of *B. burgdorferi* once it disseminates from the skin can only be studied if immunosuppression is used.[13] Both inflammation and infection are more prominent in the periphery than in the CNS.[13,14] The preferred localization in the spinal cord were the leptomeninges, nerve roots, and dorsal root ganglia.[13] For the present study, we focused on the spinal cord. We report the first comprehensive investigation of spinal cord involvement in experimental Lyme borreliosis, including both North American and European strains.

Material and methods

Borrelia Strains

The following Borrelia strains were used for these experiments: *B. burgdorferi sensu stricto* strains N40 (BbN40) and 297 (Bb297); and *B. burgdorferi* subspecies *garinii* strains Pli (BgPli), 793 (Bg793), and Pbi (BgPbi). BbN40 is a North American tick-isolate,[15] Bb297 is a CSF isolate from a patient in Connecticut.[16] BgPbi and BgPli are CSF isolates from Europe,[17] and Bg793 is a tick isolate from Europe (a gift from Martin Schriefer, Division of Vector-Borne Infectious Disease, Center for Disease Control and Prevention, Fort Collins, CO, USA).

Animals Inoculation and Necropsy

In all, 25 adult *Macaca mulatta* were inoculated intradermally with different strains of *B. burgdorferi sensu strictu* or *garinii* as follows: Bb *sensu stricto* strain N40 by needle ($N=8$) or by tick-bite ($N=4$), or strain 297 by needle ($N=2$); *B. burgdorferi garinii* strains Pbi ($N=4$), 793 ($N=2$), or Pli ($N=4$), all by needle. All NHP's inoculated by needle received 1 million spirochetes divided in seven different injection sites in the skin on the back. The majority of the NHP's were immunosuppressed with 2 mg/kg/day of dexamethasone given intramuscularly once daily either from days −7 to 28 postinoculation and then discontinued (transient immunosuppresion = TISP; $N=16$) or maintained for the duration of the experiment (permanent immunosuppresion = IS; $N=7$). Two NHP's were not given any steroids to use as nonimmunosuppressed controls. All NHP's were euthanized by intravenous injection of sodium pentotal, extensively perfused with buffer, and multiple tissues were collected at necropsy 50 days to 4 months after inoculation. The following tissues were removed at autopsy: dura-mater, brain, spinal cord, brachial plexus and median and sciatic nerves, biceps and quadriceps skeletal muscles, heart, bladder, testis, spleen, lymph nodes, and skin. Several of the tissues were cultured at time of necropsy in Borrelia culture media (BSK-II) media[18] to look for the presence of viable spirochetes. The focus of this report is on the spinal cord although some results for brain are included for comparison.

Tick Inoculation

Ixodes scapularis ticks, free of *B. burgdorferi* inherited infection, were obtained as field-collected adults from southern Connecticut (cordially provided by Durland Fish from Yale University, New Haven, CT, USA), which produced larvae for experimental use. All larvae were derived from a single cohort for the experiments described in this study. To generate infected nymphs, larvae were allowed to engorge on C3H mice that had been infected with *B. burgdorferi* for 2 weeks. Engorged larvae were collected, and then allowed to molt and harden into nymphs and tested by real-time PCR; revealing 97% were positive. The mean (s.d.) average number of spirochetes per nymph was $4.2\times10^4(3.2\times10^4)$. Prior to tick infestation (day-3), NHP's were habituated to vest. On day 0, the NHP's skin between shoulders was shaved, a tick chamber was placed, and then eight infected nymphal ticks were placed into one chamber for each monkey. The chambers were inspected on days 1, 2, and 3 to assess the number of attached ticks. If less than four ticks were attached, three extra ticks were introduced for each unfed tick. Engorged nymphs (the number of engorged nymphs per animal was in the range from 5 to 8) were collected from every animal, and were tested by real-time PCR, revealing that all of them were PCR positive with a mean (s.d.) number of spirochetes per nymph of 3.49×10^5 (2.11×10^5).

Histology

Tissues from all organs were processed for histology by routine formalin-fixation and embedding in paraffin or snap-frozen in cryomatrix (Shandon) in isopentane chilled to less than −140°C in liquid nitrogen, and for RNA extraction by storing in dry ice. Paraffin sections were cut at 5 μm and cryomatrix sections at 8 μm. Inflammation was assessed by examination of hematoxylin and eosin (H&E) staining. To compare the spirochetal load in different regions of the spinal cord, small pieces of spinal roots and meninges, dorsal root ganglia, or spinal cord free of meninges were microdissected using sterile disposable blades under a dissecting microscope by a neuropathologist (DC).

ELISA

Serum ELISA was performed as described[19] with some modifications. Each plate contained a positive control. The antigens were sonicates of the same *B. burgdorferi* strains used for inoculation. A volume of 200 μl of sonicate coating solution were added to a

microtitration plate (Linbro Scientific, Hamden, CT, USA) at a concentration of 5 μg/ml and incubated overnight at 4°C. Plates were washed three times with PBS-0.05% Tween 20, and 200 μl of monkey sera were added at a 1/5000 dilution. Sera found to be negative were run again using lower dilutions, as low as 1/500. Plates were incubated in primary antibody for 2 h at 37°C, and washed again as above. A measure of 200 μl of horseradish peroxidase-conjugated donkey antihuman immunoglobulin, isotypes G or M (Jakson Immunoresearch) were diluted 1:10 000 in PBS-Tween 20 and added to each well, and incubation was continued for 2 more hours at 37°C. Plates were washed and 200 μl of TMB-chromogen were added to each well, followed after 5–10 min by 50 μl of 8% sulfuric acid to stop the reaction. The plates were read immediately on an ELISA spectrophotometer (BioRad). The standard positive control was serum from a monkey with high titers of IgG- and IgM-specific antibody, which was run within its linear range of dilution. The cutoff for a positive ELISA was >mean plus 3 s.d. of the optical density of all preinoculation monkey sera. All serum samples were tested in duplicate.

Immunoblotting

Commercial *B. burgdorferi sensu stricto* nitrocellulose strips (Microbiology Reference Laboratory, Cypress, CA, USA) were used as previously described[10,14] according to the instructions of the diagnostic kit. The strain used for preparation of these blots was a *B. burgdorferi sensu stricto* strain CB, an isolate from an erythema migrans lesion from a patient from Valhalla, NY. Additionally, sera from the BbPbi-inoculated NHP's were also examined by immunoblot using sonicates from *B. garinii* strains by Dr Bettina Wilske (Munich, Germany). Immunoblots were considered positive based on the presence of two bands for the IgM and five bands for the IgG according to the Dressler criteria.[20]

Immunohistochemistry and Image Analysis

Immunohistochemistry was performed as previously described.[13] Endogenous peroxidase activity was reduced by incubation with 3% H_2O_2 for 10 min at room temperature. Nonspecific binding was blocked with 10% normal monkey serum (for frozen sections) or Power block (Biogenex) for paraffin sections. Rabbit polyclonal antibody antihuman IgG (Dako's A0423), IgM (Dako's A0425), Ki67 (Dako's A047), C1q (Dako's A0136), and CD3 (T-cell marker, Dako's A0452) or mouse monoclonal antibody antihuman CD20 (B-cell marker, Dako's M0755), P63 (plasma cell marker, Dako's M7077), CD38 (plasma cell marker, Dako's M7100), Ham56 (monocyte/macrophage marker, Dako's M0632) and C5b-9 (Dako's M0777) were used as primary antibody. Primary antibody for detection of *B. burgdorferi* was hyperimmune serum from a rabbit persistently infected with *B. burgdorferi* strain N40.[21] For negative controls, duplicate sections on each glass slide were incubated with affinity-purified nonspecific antibody (Sigma) matched for concentration, species, and isotype. IS heart tissue was used for C5-9 positive control. A section incubated with washing buffer instead of primary antibody was included in all assays to control for nonprimary antibody-related background. The secondary reagent was biotinylated goat anti-rabbit or goat anti-mouse polyclonal antibody (Biogenex). The tertiary reagent was horseradish peroxidase-labeled streptavidin (Biogenex). Incubation time was 30 min for the primary and 20 min for the secondary and tertiary reagents. The chromogen was 3,3 diaminobenzidine tetrahydrochloride (DAB) in 0.24% H_2O_2 for 5–15 min. The counterstaining was Mayer's hematoxylin diluted 1/5 in H_2O for 1 min. No counterstaining was used when image analysis was done. Incubations were separated by washes with Opti-Max wash buffer (Biogenex). Sections from monkey spleen or lymph nodes were used as positive controls. The intensity and extent of the immunohistochemical stains were compared by digital image analysis with Image-Pro Plus software 4.1 (Media Cybernetics). For this, a masked examiner took 3–4 digital images at $\times 40$, $\times 100$, or $\times 200$ magnification per microscopic section. The mean (s.d.) sum area (in square microns) and sum optical density (in arbitrary unit s) per microscopic field were determined and compared between groups for statistically significant differences.

RT-PCR

Total RNA was extracted with TRIzole reagent (Life Technologies) from 100 mg nonhuman primate tissue blocks. The reverse transcription (RT) was performed in 20-μl reaction volumes. RT reaction mix was 1 $\times$ RT buffer (500 mM KCl, 100 mM Tris-HCl pH 8.3), 5.5 mM $MgCl_2$, 500 μM of each dATP, dCTP, dGTP, and dTTP, 200 μM reverse specific primer (for Borrelia RT) or 1 μM oligo dT (for GAPDH or C1q RT), 0.4 U/μl RNase inhibitor, and 1.25 U MultiScribe reverse transcriptase (Perkin-Elmer). A measure of 500 ng of total RNA were used as template for all RT reactions. The only exception was RT reactions for the Borrelia standard curve used RNA extracted from a known number of spirochetes. This RNA was combined with nucleic acid from uninfected monkey tissue. The RT reactions were incubated at 48°C for 40 min followed by 5 min at 95°C.

PCR amplication of *Macaca mulatta* genes was carried out in 25 μl reaction mixtures containing 1 $\times$ PCR buffer without magnesium, 2.5 U Taq DNA polymerase (5 U/μl, Invitrogen), 200 μM dNTP, 2 mM $MgCl_2$ and 0.5 μM of each forward and reverse specific primers. The PCR mixture was prepared

before the addition of 1 μl (100 ng) of cDNA template. The forward and reverse primers for C1q corresponded to the published human C1q beta chain sequences 349–369 (5′-CTGGCTAGAC CATGGTGAGTT-3′) (forward primer) and 862–881 (5′-AAGATGCTGTTGGCACCCTC-3′) (reverse primer) (gene bank accession No. X03048), with a predicted PCR fragment size of 532 bp. The forward and reverse primers for GAPDH corresponded to the published human GAPDH sequences 86–110 (5′-TGAAGGTCGGAGTCAACGGATTTGG-3′) (forward primer) and 461–483 (5′-GTTCACACCCATGAC GAACATGG-3′) (reverse primer) (gene bank accession number gi 7669491), with a predicted fragment size of 397 bp. PCR amplification was carried out using in a Gene Amp PCR system 9700 (PE Applied Biosystems). The amplification program consisted of an initial denaturing step at 94°C for 5 min. The remaining cycles were 1 min at 94°C, 30 s at 58°C and 30 s at 72°C. The number of cycles performed was 40. Final extension was 5 min at 72°C.

DNA Sequencing

After RT-PCR amplification, the PCR products of *M. mulatta* C1q and GAPDH were purified using the QIAquick PCR Purification Kit (QIAGEN Cat# 28104) and sequenced after TA cloning (for GAPDH) or by cycle sequencing (for C1q) by ABI BigDye terminator chemistry on an ABI3100 Genetic Analyzer at the University of Medicine and Dentistry of New Jersey Molecular Research Facility. The human and nonhuman primate sequences were compared using BLAST II software (NCBI).

TaqMan RT-PCR

Taqman probes and primers were designed with Primer express software (Perkin-Elmer Applied Biosystems, Foster City, CA, USA). For Borrelia TaqMan, multiple 16S rRNA Lyme disease and relapsing fever Borrelia sequences available in GeneBank were aligned and the primers chosen to target a 136 bp long segment common to many Borrelia spp. The forward primer corresponds to *B. burgdorferi* B31 16S rRNA sequence 739–760 (5′-GGTCAAGACTGACGCTGAGTCA-3, GeneBank accession number U03396). The reverse primer corresponds to sequence 874–853 (5′-GGCGGCACAC TTAACACGTTAG-3′), and the fluorogenic probe to sequence 801–829{(6FAM) 5′-TCTACGCTGTAAAC GATGCACACTTTGGTG-3′(TAMRA)}. Taqman PCR reactions were performed in 50 μl volumes. TaqMan PCR reaction mix was 1 × Taqman Universal PCR Master Mix (PE Biosystems PN.4304437), 100 μM 16S probe, 200 μM forward 16S primer, 200 μM 16S reverse primer, and 10 μl cDNA as template. Samples were run in duplicate. Amplification and detection were performed with an ABI 7700 system (Perkin-Elmer Applied Biosystems) with the following cycling conditions: UNG incubation 50°C for 2 min, AmpliTaq gold activation 95°C for 5 min, 40 cycles of denaturation at 95°C for 15 s and annealing/extending at 60°C for 1 min. For quantification of the spirochetal load, *B. burgdorferi* strain N40 spirochetes were cultured *in vitro* and counted in a Petroff–Hausser chamber by phase-contrast microscopy. RNA was extracted from known numbers of spirochetes, added to RNA extracted from skeletal muscle from noninfected NHP to control for the inhibitory effect of host DNA, and used in log10 dilutions to get a standard curve with a liner range coefficient of $\geq$0.95. TaqMan reactions with H_2O instead of tissue cDNA were included as negative controls. A Ct of 40 and similar duplicate values for each sample was required for the assay to be valid.

For *M. mulatta* GAPDH TaqMan, the forward and reverse primers were 5′-GCGAGATCCCTCCAAAAT CA-3′ and 5′-CCAGTGGACTCCACGACGTA-3′ respectively, and the fluorogenic probe was [VIC (5′-GTGGGGCGATGCTGGCGCT-3′)] (Figure 6). For C1q TaqMan, the following nonhuman primate-specific primers and probe were designed based on partial sequencing of *M. mulatta* C1q (Figure 6): forward primer corresponding to sequence 146–160 (5′-GCGCCGGGACCAGAC-3′), reverse primer corresponding to sequence 180–204 (5′-CGTAGTTGTTG TTCATGTTGGTGAT-3′), and flurogenic probe corresponding to sequence 162–178 [6 FAM (5′-ATCCG CTTCGACCACGT-3′) TAMRA]. The corresponding sequences on the human C1q B chain (gi29537) were 328–342, 362–386, and 344–360, respectively. The RT and TaqMan cycling conditions for GAPDH and C1q were the same as for Borrelia TaqMan.

Statistical Analysis

For digital image analysis, differences in mean sum density or area were compared for statistical significance using nonparametric tests (Mann–Whitney test) with SPSS software version 10. The *P*-values lower than 0.05 were considered significant. Nonparametric tests were also used to compare mean delta Ct values for C1q TaqMan RT-PCR.

Results

Animal Infections

In all, 25 nonhuman primates (NHP's) (*Macaca mulatta)* inoculated with different strains of *Borrelia burgdorferi* were used for these studies (Table 1). Of the five *B. burgdorferi* strains used, two were *B. burgdorferi sensu stricto* from North America (N40 and 297) and three were *B. burgdorferi garinii* from Europe (Pbi, 793, and Pli). All NHP's were inoculated intradermally by needle injection except for four NHP's inoculated with *B. burgdorferi* strain N40 by tick-bite. Only two of the NHP's were immunocompetent (IC) for the duration of the

Table 1 Inoculation of nonhuman primates with different degree of immunosuppression with *Borrelia burgdorferi* (Bb) *sensu stricto* strains N40 or 297 or *garinii* (Bg) strains Pbi, 793, or Pli by needle or tick-bite

Strain	*ID No.*	*Inoculation*	*Necropsy (weeks)*	*Immunosuppression*	*αBb ELISA*[a]	*αBb IgM WB*	*αBb IgG WB*	*Inflammation*[b]
BbN40	M14	Needle	24	None	+	+	+	−
	M15	Needle	24	None	+	+	+	−
	099	Needle	13	Transient[c]	+	+	+	−
	177	Needle	13	Transient	+	+	+	−
	199	Needle	13	Transient	+	+	+	−
	383	Needle	13	Transient	+	+	+	−
	154	Tick	13	Transient	+	+	+	−
	192	Tick	13	Transient	+	+	+	−
	211	Tick	13	Transient	+	+	+	−
	242	Tick	13	Transient	+	+	+	−
	652	Needle	13	Permanent[d]	+	+	−	−
	372	Needle	7	Permanent	+	NA	NA	−
	794	Needle	7	Permanent	+	NA	NA	−
Bb297	571	Needle	7	Permanent	+	+	+	−
	708	Needle	7	Permanent	−	−	−	−
BgPbi	321	Needle	17	Transient	+/−[e,f]	−	−	+
	105	Needle	17	Transient	+[f]	−	−	−
	981	Needle	7	Permanent	−	−	−	−
	012	Needle	7	Permanent	−	−	−	−
Bg793	111	Needle	17	Transient	+[f]	−	−	−
	985	Needle	17	Transient	+[f]	−	−	−
BgPli	558	Needle	7	Transient	−	−	−	−
	608	Needle	7	Transient	−	−	−	−
	673	Needle	13	Transient	+	+	+	NA[g]
	314	Needle	13	Transient	+	+	+	NA

[a]Measured by ELISA with homologous sonicate and serum diluted 1:5000.
[b]Only NHP ID. 321 showed meningoradiculitis by standard H&E examination.
[c]Transient immunosuppresion for 3 weeks.
[d]Constant immunosuppression for 2 (372.794) to 3 (652) months.
[e]ELISA positive when serum diluted 1:500 but not when diluted 1:5000.
[f]IgM only.
[g]NA = not available.

experiment. All the other NHP's[23] were immunosuppressed with dexamethasone beginning a few days prior to inoculation to increase the probability of developing infection. Dexamethasone was given either transiently (TISP) during the first month of infection ($N = 14$) or permanently (IS) for the duration of infection (2–4 months) ($N = 7$). None of the NHP's developed erythema migrans or any signs of neurological disease. Serial examination of cerebrospinal fluid (CSF) failed to show any evidence of CSF-leukocytosis. Viable spirochetes were identified only in tissue cultures from IS-NHP's. None of the cultures from TISP or IC-NHP's were positive for spirochetes.

Antibody Response

Serum ELISA with homologous whole–cell sonicates showed that all NHP's inoculated with *B. burgdorferi. sensu stricto* strain N40 (BbN40) either by syringe or by tick developed specific antibody (Table 1). In contrast, one out of two NHP's inoculated with *B. burgdorferi* strain 297 (Bb297) and four out of 10 NHP's inoculated with *B. burgdorferi garinii* strains had no detectable anti *B. burgdorferi* antibodies by ELISA. Table 1 shows a summary of the WB results. The Western blot results for most of the NHP's inoculated with strain BbN40 have been previously reported.[12] All IC and TISP-NHP's inoculated with BbN40 had positive IgM and IgG WB. Only some of the WB for the IS NHP's inoculated with BbN40 or Bb297 were positive. In contrast, all WB for the BgPbi and Bg793 NHP's were negative. The only NHP's inoculated with *B. garinii* strains that had positive WB were the two Pli-inoculated NHP's Nos. 673 and 314. It was possible that the negative WB in NHP's inoculated with the *garinii* strains was a false-negative result due to the use of WB strips made with a North American *sensu stricto* strain. To investigate this possibility, we asked Dr Bettina Wilske to run WB on necropsy sera from the BgPbi-inoculated NHP's using WB strips prepared with *garinii* strains. These results were also negative (not shown).

Inflammation in the Brain and Spinal Cord

Microscopic examination of H&E-stained brain and spinal cord frozen and paraffin-embedded sections from brain, brainstem and cerebellum, and cervical, thoracic, lumbosacral cord, and cauda failed to reveal any significant inflammation with the only

exception of NHP 321, a transiently immunosuppressed NHP (TISP) inoculated with *Bb. garinii* strain Pbi (Table 1). The inflammatory infiltrate was a mild mononuclear meningoradiculitis, found along the entire neuroaxis including the brain, brainstem, spinal cord, spinal nerve roots (Figure 1a,b) and dorsal root ganglia (Figure 1h). To rule out the possibility that this meningoradiculitis was not due to Borrelia infection but to some other pathogen, we had tissue sections and nucleic acid extracts from the spinal cords of NHP's 321 and 105 examined by immunohistochemistry and TaqMan RT-PCR. We chose to look for monkey CMV and Herpes B, the two most likely pathogens to cause such reaction.[22] No evidence of CMV or Herpes B was found in either monkey (Dr Peter Barry, personal communication).

To characterize the inflammatory infiltrate in NHP No. 321, we did immunohistochemistry with specific antibodies to CD3 (T cell), CD20 (B cells), HAM56 (macrophages), and P63 (plasma cells). As a noninflamed control, we used tissues from NHP

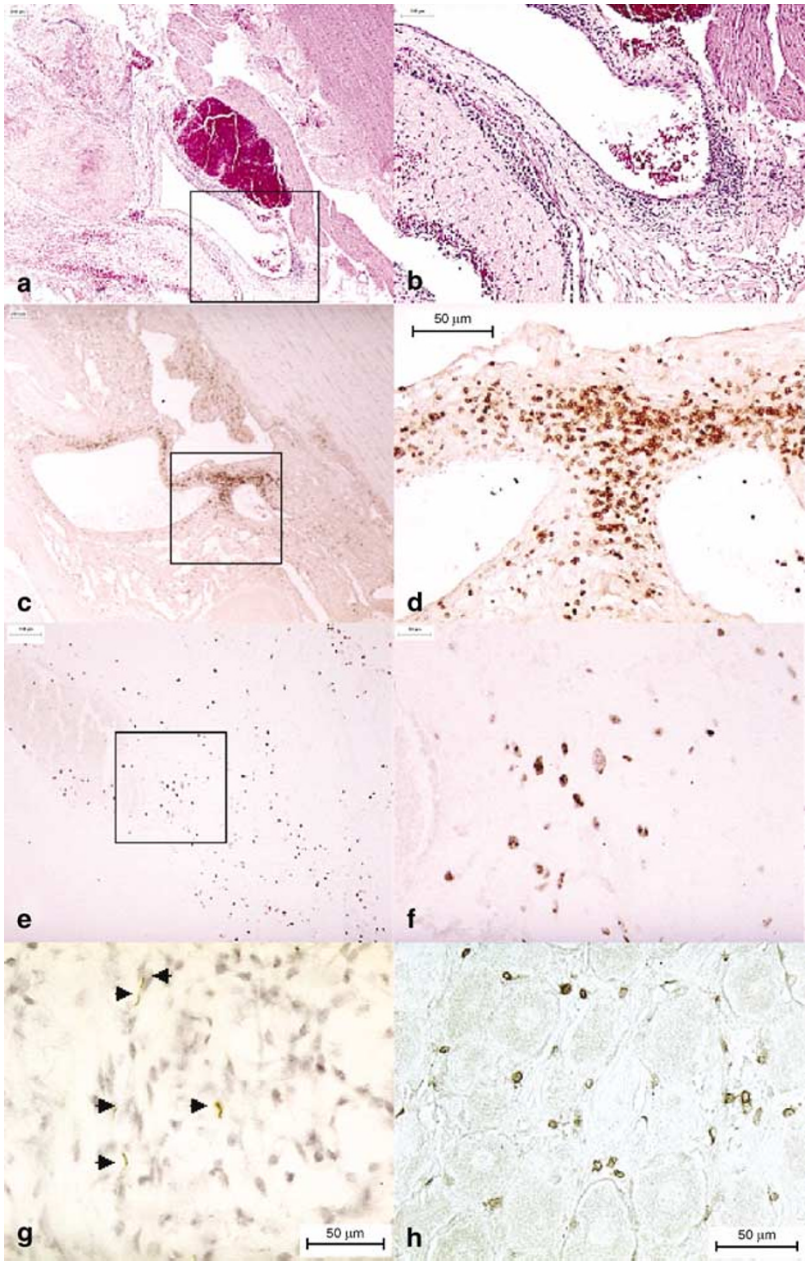

Figure 1 Infection and inflammation in the spinal cord of nonhuman primates inoculated with *B. burgdorferi*: Panels (**a**) (×40 magnification) and (**b**) (×100) show hematoxylin and eosin staining of the lumbar spinal cord from TISP NHP 321; panels (**c**) (×100) and (**d**) (×400) show CD3 immunostaining in the lumbosacral spinal cord of TISP NHP 321; panels (**e**) (×100) and (**f**) (×400) show Ki67 immunostaining in lumbosacral spinal cord of TISP NHP 321; panel (**g**) (×400) shows five spirochetes (arrows) in one dorsal root ganglia from IS NHP 372; panel (**h**) (×400) shows CD3-positive cells infiltrating the dorsal root ganglia of TISP NHP 321.

105, also inoculated with *B. garinii* strain Pbi but negative for inflammation by H&E staining. The predominant cells of the inflammatory infiltrate were T cells and plasma cells. Infiltrates of T cells were observed in the leptomeninges, spinal motor, and sensory roots (Figure 1c and d), and in the dorsal root ganglia (Figure 1h) in NHP 321 but not in NHP 105. B cells and macrophages were rare. H&E staining showed the presence of frequent plasma cells but only in HNP 321. To further investigate this, we did immunohistochemistry using a primary antibody against the endoplasmic reticulum protein P63 (Dako).[13] Although this antibody is specific for plasma cells among lymphoid cells, it also labels neurons and glial cells. Therefore, P63 immunostaining were useful only in the leptomeninges and nerve roots but not in the cord parenchyma. Digital image analysis of P63 immunostaining of the leptomeninges and nerve roots of the lumbosacral spinal cord of NHP 321 compared with NHP 105 revealed significantly increased P63 signal in NHP 321 compared with 105: the mean (s.d.) density was 19 695(7397) and 329 (336) for 321 and 105, respectively ($P<0.001$).

To compare the extent of the inflammatory infiltrate at various levels of the spinal cord of NHP 321, we used digital image analysis of tissue sections immunostained for the cellular proliferation maker Ki67. Ki67 (Dako's M7187) is a protein preferentially expressed during G1, S, G2, and M phases of the cell cycle and was found increased in the spinal cord of NHP 321 (Figure 1e and f). The results (Figure 2) showed significantly increased Ki67 at all levels of the spinal cord and the brainstem of NHP 321 compared with noninflamed control 105 ($P<0.03$ for cervical cord; $P<0.01$ for brain stem, thoracic cord, and lumbosacral cord).

Deposition of Immunoglobulin and Complement in Spinal Cord

H&E examination indicated plasma cells were present in the meningoradiculitis that develop in NHP 321. Since immunoglobulin production is the primary function of plasma cells, we next investigated whether there was accumulation of antibody and complement in the spinal cord of NHP 321 in comparison with NHP 105 as a noninflamed control. Light microscopic examination of immunostained sections revealed increased deposition of IgM, IgG, and C1q in the meninges and nerve roots of the spinal cord of NHP 321 (Figure 3a, d, and g) in comparison with NHP 105 (Figure 3c, f, and i). Immunostaining with negative control antibody gave negative results (Figure 5b, e, and h), confirming the specificity of the stains. The intensity of the staining appeared higher for IgG than IgM or C1q. However, immunostaining for membrane attack complement (MAC) was negative (not shown).

To compare the extent of antibody deposition, we did digital image analysis of paraffin sections immunostained with antibody to IgM or IgG in lumbosacral spinal cord from NHP's 321 and 105 (Figure 4). The mean density of IgM per $\times 40$ microscopic field was 2.2×10^5 and 1×10^5, respectively, for NHP's 321 and 105, respectively ($P=0.001$). Similarly, the mean density of IgG per $\times 40$ microscopic field was 5.8×10^5 and 4.9×10^5 in NHP's 321 and 105, respectively ($P<0.0001$). Digital image analysis of C1q deposition at various levels of the spinal cord and brainstem of NHP's 321 and 105 also revealed significantly increased amounts in NHP 321 at brainstem and cervical cord levels ($P<0.05$) (Figure 5).

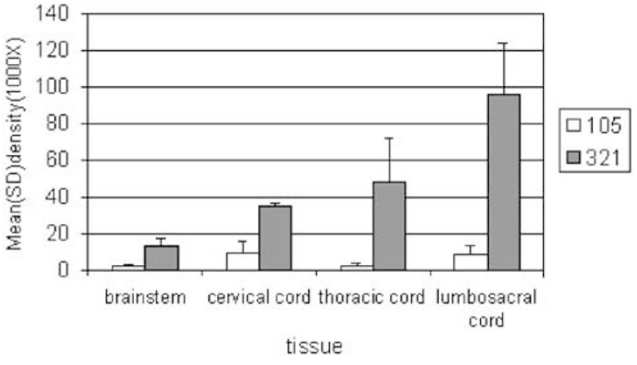

Figure 2 Digital image analysis of Ki-67 immunostaining in the brainstem and spinal cord of BgPbi-inoculated TISP NHP's 105&321.

Synthesis of C1q in the CNS

Immunohistochemistry indicated one of the consequences of Lyme meningoradiculitis was increased presence of the first component of the complement cascade, C1q, in inflamed spinal cord tissues. To investigate whether C1q was produced locally in the central nervous system and not only deposited from the periphery, we measured C1q mRNA in the brain and spinal cord by TaqMan RT-PCR in the various groups of NHP's. As a TaqMan RT-PCR house-keeping gene control we measured GAPDH. Since the sequence of the *Macaca mulatta* GAPDH and C1q genes was not known, we first obtained partial sequences of both genes. For this, we extracted *M. mulatta* mRNA from skeletal muscle and did RT-PCR using forward and reverse primers based on published C1q and GAPDH sequences for *Homo sapiens*. The results (Figure 6) showed that both the GAPDH and C1q sequences from *M. mulatta* were very similar to the *Homo sapiens* sequences. The percent identity for GAPDH was 97% (390/398) and for C1q was 96% (378/393).

These *M. mulatta* sequences were used to design forward and reverse primers and probes used for quantitation of C1q expression by TaqMan RT-PCR. Results were expressed as 'Delta Ct' values, corresponding to the difference of the Ct values for C1q and GAPDH TaqMan assays using total RNA from

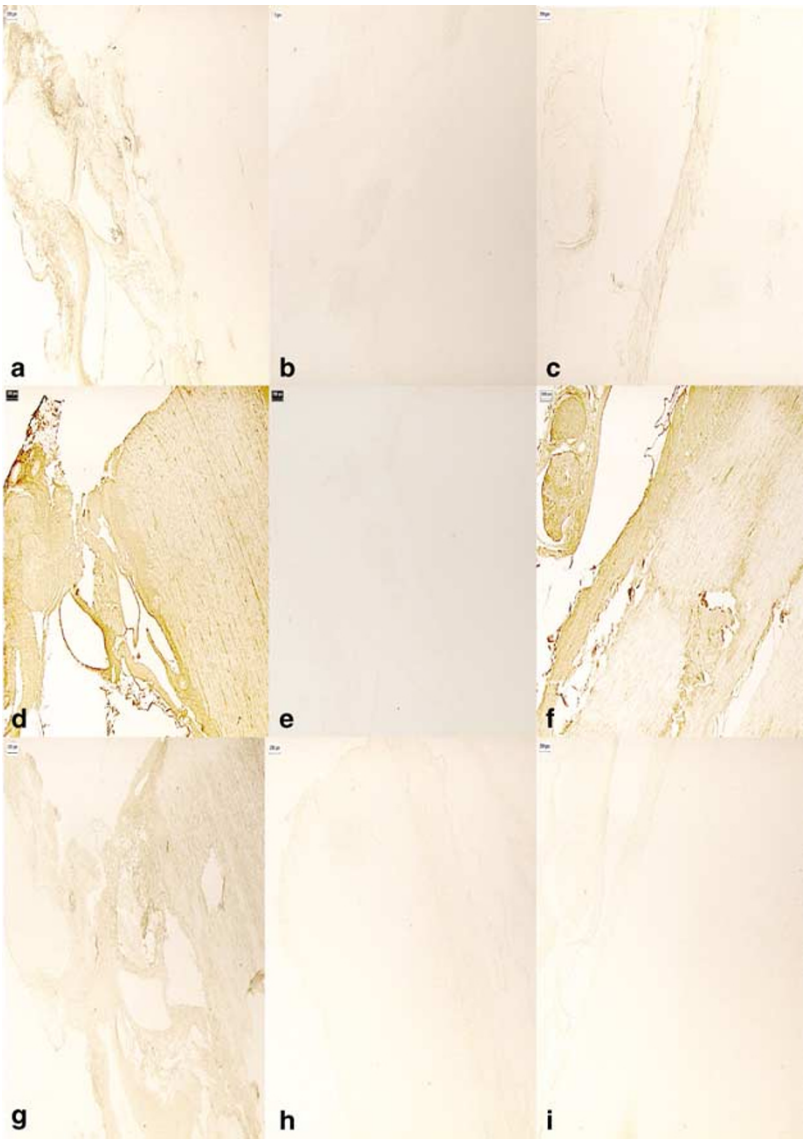

Figure 3 IgM (**a**, **b**, **c**), IgG (**d**, **e**, **f**), and C1q (**g**, **h**, **i**) immunostaining in lumbosacral spinal cord from NHP's 321 (left and center panels) and 105 (right panel) after incubation with primary antibody (left and right panels) or negative control antibody (center panels) (× 20 magnification).

the same tissue block. Using this method, the higher the amount of input C1q mRNA, the lower the delta Ct value. The results (Figure 7) showed the spinal cord (a) and brain (b) of NHP 321 had significantly more C1q mRNA than any of the other NHP's examined ($P<0.0001$ for both spinal cord and brain). These results not only confirmed C1q is increased in the brain and spinal cord in Lyme meningoradiculitis but also demonstrated it is locally produced in inflamed areas of the CNS.

Measurement of the Spirochetal Load in Spinal Cord

The finding of specific antibody in serum from 20 of the 25 NHP's inoculated with various strains of *B. burgdorferi* indicated the majority had been exposed to Lyme disease borrelias. To investigate whether borrelias was still present in the spinal cords removed at necropsy, we examined the tissues for the presence of spirochetes by immunohistochemistry and TaqMan RT-PCR. Microscopic examination of spinal cord sections immunostained with hyperimmune rabbit serum specific to *B. burgdorferi* strain N40 (which cross-reacts with *garinii* strains) revealed the presence of spirochetes only in tissues from permanently immunosuppressed NHP's (IS NHP's). The localization was in the duramater, leptomeninges, dorsal root ganglia (Figure 1g) and motor and sensory spinal roots but not in the cord parenchyma itself.

In no case were spirochetes observed microscopically in the spinal cord of immunocompetent (IC) or

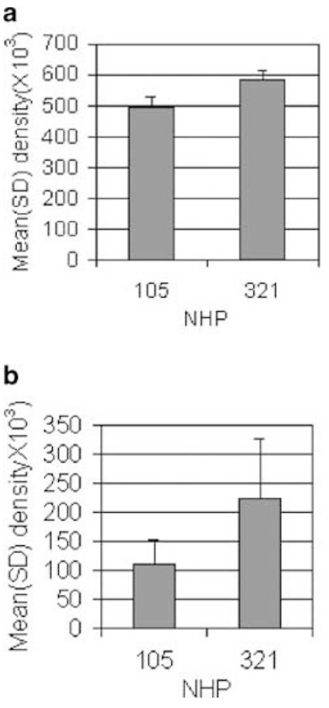

Figure 4 Digital image analysis of sum density for IgG (panel (**a**)) and IgM(panel (**b**)) immunostaining in lumbosacral spinal cord from BgPbi-inoculated NHP's 321& 105.

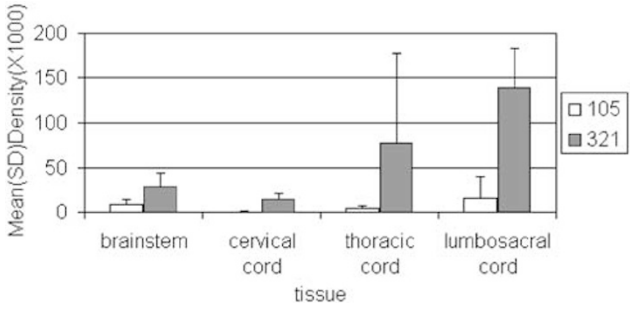

Figure 5 Digital image analysis of sum density for C1q-IHC in brain and spinal cord of Bg Pbi-inoculated NHP's 321&105.

transiently immunosuppressed (TISP) NHP's. The implication was that if IC or TISP NHP's had any persistent infection the spirochetal load was very low. We examined this possibility using a very sensitive TaqMan RT-PCR specific for the 16S rRNA of Borrelia spp. This assay included a standard curve for quantitation of the spirochetal load and is sensitive in the range of 10^0–10^6 spirochetes per 500 ng host nucleic acid (D. Cadavid *et al*, unpublished results). The results showed that none of the spinal cord tissues from IC or TISP NHP's had any detectable spirochetes in the spinal cord (Table 2). In contrast, all the cervical, thoracic, and lumbosacral spinal cord tissues examined from BbN40-inoculated IS NHP's were positive for spirochetes, although the spirochetal load was quite variable

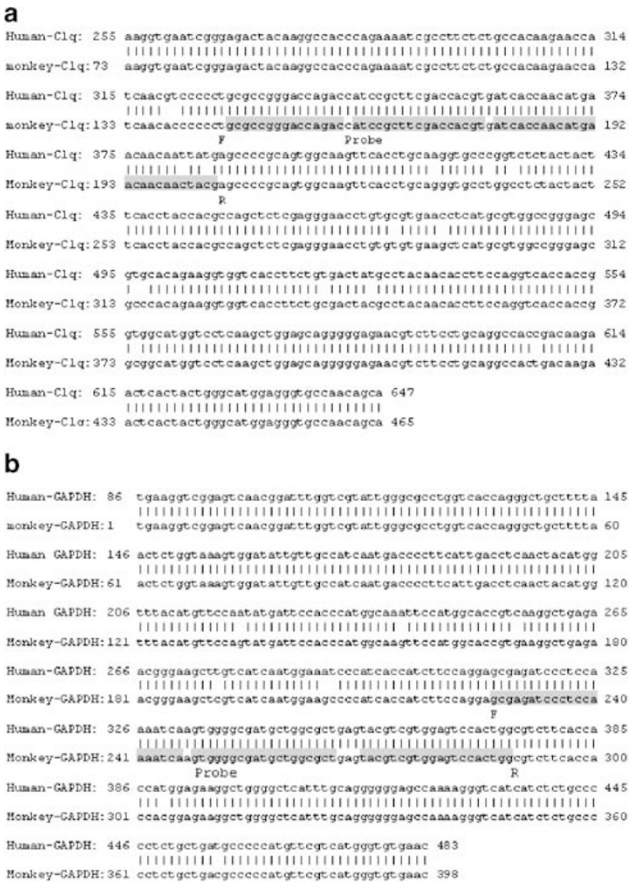

Figure 6 Alignment of DNA sequences from *Macaca mulatta* C1q beta chain (lower panel) and glyceraldehydes-3-phosphate dehydrogenase (upper panel) in comparison with the corresponding sequences for *Homo sapiens*. Forward and reverse primers and probes used for TaqMan RT-PCR are shown for each gene.

(from as high as 10 million in one lumbosacral cord to as low as 47 in one thoracic cord). One of the Bb297-IS NHP's (ID No. 571) also had large numbers of spirochetes in the spinal cord. In contrast, the spirochetal load in the spinal cord of BgPbi-inoculated IS NHP's was very low (Table 2).

Both the immunohistochemical examination and the TaqMan RT-PCR analysis suggested great variability in the spirochetal load not only between individual animals but also between different regions of the spinal cord. To further investigate this, we compared the spirochetal load by TaqMan RT-PCR in spinal roots and leptomeninges with dorsal root ganglia and spinal cord parenchyma that were microdissected from the cervical and thoracic spinal cords from all three BbN40-inoculated IS-NHP's (Table 3). The results confirmed that the majority of spirochetes were in the meninges and spinal roots and the dorsal root ganglia rather than in the cord parenchyma itself. For all three NHP's combined, the ratio of spirochetes in the leptomeninges, nerve roots and dorsal root ganglia to cord parenchyma varied between 12 and 185 to 1 (mean ratio was 100).

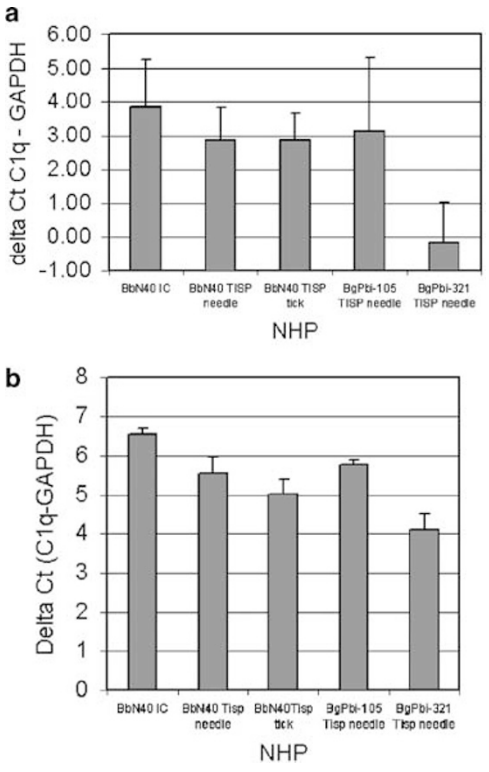

Figure 7 Measurement of C1q mRNA by TaqMan RT-PCR in spinal cord (panel (**a**)) and brain (panel (**b**)) of NHP's with different degree of immunosuppression inoculated with *B. burgdorferi sensu stricto* strain N40 or *garinii* strain Pbi.

Discussion

This manuscript presents the first comprehensive study of spinal cord involvement in experimental Lyme borreliosis. The paucity of neuroborreliosis in small animal models of Lyme disease has hampered our understanding of its pathogenesis. Using NHP's with various degrees of immunosuppression, we were able to study the localization of spirochetes in the spinal cord as well as the inflammatory reaction of the host to the infection. The main findings of these studies were as follows: (1) Meningoradiculitis occurs in nonhuman primates (NHPs) inoculated with *B. burgdorferi* but it is infrequent. (2) The inflammatory infiltrate in the nervous system of the affected animal was mild, but present throughout the neuroaxis and localized to the leptomeninges, nerve roots and the dorsal root ganglia. (3) The predominant cells of the inflammatory infiltrate were T cells and plasma cells. (4) Meningoradiculitis was accompanied by significant presence of IgG, IgM, and C1q but not MAC (C5-9), mostly in the meninges and nerve roots. (5) C1q was produced in the brain and spinal cord and increased in inflamed tissues. (6) Large numbers of spirochetes were found outside of the spinal cord during immunosuppression (dorsal root ganglia and dura mater) rather than in the cord parenchyma itself, but they become undetectable once immunocompetence is restored.

Table 2 Spirochetal load in the spinal cord of nonhuman primates with different degree of immunosuppression 2–4 months after inoculation by needle or tick-bite with different strains of *Borrelia burgdorferi* subspecies *sensu stricto* (BbN40 or 297) or *garinii* (BgPbi, 793, or Pli)

Inoculation	*ID No.*	*Immunosuppression*	*Mean (s.d.) borrelias per 100 mg tissue*[a]		
			Lumbosacral Cord	*Cervical cord*	*Thoracic cord*
BbN40-needle	M14	None	ND[b]	0	ND
	M15	None	ND	0	ND
	099	Transient	ND	0	ND
	177	Transient	ND	0	ND
	199	Transient	ND	0	ND
	383	Transient	ND	0	ND
	652	Permanent	6 920(10,562)	290(289)	47(106)
	372	Permanent	10 950 500(13 316 097)	129 264(151 029)	78 675(78 910)
	794	Permanent	50 814(57 851)	4 022(2 544)	1 604(1 017)
BbN40-tick	154	Transient	ND	0	ND
	192	Transient	ND	0	ND
	211	Transient	ND	0	ND
	242	Transient	ND	0	ND
Bb297-needle	571	Permanent	0	4987(4990)	2597(3672)
	708	Permanent	0	0	0
BgPbi-needle	321	Transient	0	0	0
	105	Transient	0	0	0
	981	Permanent	50	0	0
	012	Permanent	60	0	275(389)
Bg793-needle	985	Transient	0	0	0
	111	Transient	0	0	0
BgPli-needle	558	Transient	0	0	0
	608	Transient	0	0	0
	673	Transient	0	0	0
	314	Transient	0	0	0

[a]By TaqMan RT-PCR of *Borrelia* spp. 16S rRNA.
[b]ND = not done.

Table 3 Spirochetal load in microdissected areas of the spinal cord of immunosuppressed nonhuman primates (NHPs)[a] inoculated with *B. burgdorferi* strain N40 by needle

ID no.	*Tissue*	*Mean borrelias (× 10²) per 100 mg tissue RNA*[b]		
		Meninges & nerve roots	*Dorsal root ganglia*	Cord parenchyma
794	Cervical cord	370	NA	2
	Thoracic cord	115	NA	0
372	Cervical cord	2655	3827	38
	Thoracic cord	4771	NA	29
652	Cervical cord	12	54	0
	Thoracic cord	0	NA	0

[a]Necropsy 2 months after inoculation for NHP's 794 and 372 and 3 months after inoculation for NHP ID. 652.
[b]Measured by Taqman RT-PCR amplification of the *Borrelia* spp.16 s rRNA gene.

Inoculation of BbN40 either by needle or tick resulted in infection in 14/15 NHP's. In contrast, the evidence that the NHP's inoculated with the various *garinii* strains was less clear. Only 2/10 animals had clear serological evidence of infection (No. 673 and 314). In one of these NHP' both PCR and immunohistochemistry revealed the presence of spirochetes in the skin and peripheral nerves (not shown). We also conclude that NHP 321 was also infected based on the finding of inflammation (meningoradiculitis) and the heart (not shown). Meningoradiculitis as seen in NHP 321 resembles the Banwarth's syndrome of European Lyme borreliosis. In one large European series of over a thousand cases the second most common manifestation of Lyme borreliosis after erythema migrans ($N=458$) was meningopolyradiculoneuritis ($N=404$).[23] In comparison, arthritis ($N=63$), acrodermatitis chronica atrophicans ($N=72$), carditis ($N=13$), and lymphadenosis benigna cutis ($N=5$) were less common.[23] CNS parenchymal complications (encephalomyelitis) only develop in about 0.1% of patients with Lyme borreliosis. MRI of the spinal cord done in a handful of patients has demonstrated focal or diffuse areas of increased signal most often in the cervical cord.[24–28] In one case myelography revealed lumbar arachnoiditis.[29]

There can be different explanations for the development of meningoradiculitis in NHP 321. One is that it was unrelated to Borrelia inoculation and caused by another pathogen, like some primate virus. To rule out this possibility, we had tissues from both NHP's 321 and 105 studied by TaqMan and immunohistochemistry for CMV and Herpes B, the two most common primate pathogens that may involve the spinal cord. The results showed no evidence of CMV or Herpes B infection in either animal. What we believe is a more likely scenario is that NHP 321 developed meningoradiculitis as a result of inoculation with *B. garinii*. The spirochete likely reached the spinal cord during the initial phase of immunosuppression (which lasts about 4 weeks in a TISP NHP like 321) but once the immunosuppression was discontinued the infection resolved spontaneously or was eradicated by the immune system. The finding of specific antibody by ELISA supports this (Table 1).

Much of the variability that we observed from animal to animal may be the result of working with an outbred population of experimental animals. This may explain why only two of the four NHPs inoculated with BgPli and one of the two NHPs inoculated with Bb297 developed specific antibody (Table 1). However, the uniform results obtained with the TISP NHP's inoculated with BbN40 argues against this possibility. Another factor that may have contributed to the variability is differences in the tropism for the spinal cord of *M. mulatta* of the various *B. burgdorferi* strains. Significant differences in neurotropism have been demonstrated between strains and serotypes of relapsing fever borrelias.[7,30] Finally, the use of nonclonal populations of spirochetes may have contributed to the variability of the results.

Our finding of increased deposition of IgM, IgG, and C1q in inflamed spinal cord is similar to what has been observed in skeletal muscle of NHP's inoculated with *B. burgdorferi*.[31] The deposition of IgM, IgG, and C1q indicates activation of the humoral arm of the immune response to the infection, and implies a potential mechanism of tissue injury. Prior studies suggest an important role for complement in the pathogenesis of Lyme borreliosis. Roberts *et al*[32] reported increased C1q-binding immune complexes in serum from rhesus macaques infected with *B. burgdorferi*. C1q is not only able to activate the complement pathway, but when presented in proper fashion also might induce the production of chemokines and cytokines (IL-6, IL-8) that could contribute to the acute-phase response and recruitment of inflammatory cells in Lyme borreliosis.[33] An important role for C1q has also been suggested in the pathogenesis of other neurological disorders affecting the brain and the spinal cord including amyotrophic lateral sclerosis,[34] traumatic nerve injury,[35] prion encephalophathies,[36] and viral and autoimmune encephalomyelitis.[37]

The factors responsible for development of Lyme neuroborreliosis in humans are still poorly understood. Future studies are needed to better understand if persistent low-level infection and inflammation may explain why some patients with Lyme disease who are not promptly and adequately treated with antibiotics[38] or even after they are[39] continue to have symptoms.

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References

1 Steere AC. Lyme disease [see comments]. N Engl J Med 2001;345:115–125.
2 Pachner AR, Cadavid D. Lyme neuroborreliosis. In: Griggs RC, Joynt RJ (eds). Clinical Neurology on CD ROM. Lippincott-Raven: Philadelphia, 2002.
3 Bannwarth A. Chronische lymphocytäre Meningitis, entzündliche Polyneuritis und Rheumatismus. Arch Psychiatr Nervenkr 1941;113:284–376.
4 Bannwarth A. Zur Klinic und Pathogenese der 'chronischen lymphocytären Meningitis'. Arch Psychiatr Nervenkr 1944;117:161–185.
5 Duray PH. The surgical pathology of human Lyme disease. An enlarging picture. Am J Surg Pathol 1987;11(Suppl 1):47–60.
6 Busch U, Hizo-Teufel C, Boehmer R, *et al.* Three species of *Borrelia burgdorferi sensu lato* (*B. burgdorferi sensu stricto, B. afzelii, and B. garinii*) identified from cerebrospinal fluid isolates by pulsed-field gel electrophoresis and PCR. J Clin Microbiol 1996;34:1072–1078.
7 Cadavid D, Barbour AG. Neuroborreliosis during relapsing fever: review of the clinical manifestations, pathology, and treatment of infections in humans and experimental animals. Clin Infect Diseases 1998;26:151–164.
8 Pachner AR, Delaney E, O'Neill T, *et al.* Inoculation of nonhuman primates with the N40 strain of *Borrelia burgdorferi* leads to a model of Lyme neuroborreliosis faithful to the human disease. Neurology 1995;45: 165–172.
9 Pachner AR, Zhang WF, Schaefer H, *et al.* Detection of active infection in nonhuman primates with Lyme neuroborreliosis: comparison of PCR, culture, and a bioassay. J Clin Microbiol 1998;36: 3243–3247.
10 Pachner AR, Amemiya K, Bartlett M, *et al.* Lyme borreliosis in rhesus macaques: effects of corticosteroids on spirochetal load and isotype switching of antiborrelia burgdorferi antibody. Clin Diagn Lab Immunol 2001;8:225–232.
11 Pachner AR, Delaney E, O'Neill T. Neuroborreliosis in the nonhuman primate: *Borrelia burgdorferi* persists in the central nervous system [see comments]. Ann Neurol 1995;38:667–669.
12 Pachner AR, Dail D, Li L, *et al.* Humoral immune response associated with lyme borreliosis in nonhuman primates: analysis by immunoblotting and enzyme-linked immunosorbent assay with sonicates or recombinant proteins. Clin Diagn Lab Immunol 2002;9:1348–1355.
13 Cadavid D, O'Neill T, Schaefer H, *et al.* Localization of *Borrelia burgdorferi* in the nervous system and other organs in a nonhuman primate model of lyme disease. Lab Invest 2000;80:1043–1054.
14 Pachner AR, Cadavid D, Shu G, *et al.* Central and peripheral nervous system infection, immunity, and inflammation in the NHP model of Lyme borreliosis. Ann Neurol 2001;50:330–338.
15 Moody KD, Barthold SW, Terwilliger GA. Lyme borreliosis in laboratory animals: effect of host species and *in vitro* passage of *Borrelia burgdorferi*. Am J Trop Med Hyg 1990;43:87–92.
16 Masuzawa T, Okada Y, Yanagihara Y. Protective activity of antisera against isolates of *Borrelia burgdorferi* from various geographical origins. Microbiol Immunol 1993;37:79–83.
17 Wilske B, Busch U, Eiffert H, *et al.* Diversity of OspA and OspC among cerebrospinal fluid isolates of *Borrelia burgdorferi sensu lato* from patients with neuroborreliosis in Germany. Med Microbiol Immunol (Berl) 1996;184:195–201.
18 Barbour AG. Isolation and cultivation of Lyme disease spirochetes. Yale J Biol Med 1984;57:521–525.
19 Pachner AR, Delaney E, Zhang WF, *et al.* Protection from Lyme neuroborreliosis in nonhuman primates with a multiantigenic vaccine. Clin Immunol 1999;91:310–313.
20 Dressler F, Whalen JA, Reinhardt BN, *et al.* Western blotting in the serodiagnosis of Lyme disease. J Infect Dis 1993;167:392–400.
21 Pachner AR, Braswell ST, Delaney E, *et al.* A rabbit model of Lyme neuroborreliosis: characterization by PCR, serology, and sequencing of the OspA gene from the brain. Neurology 1994;44:1938–1943.
22 Lockridge KM, Sequar G, Zhou SS, *et al.* Pathogenesis of experimental rhesus cytomegalovirus infection. J Virol 1999;73:9576–9583.
23 Schmidt R, Kabatzki J, Hartung S, *et al.* Erythema migrans borreliosis in the Federal Republic of Germany. Epidemiology and clinical aspects]. Dtsch Med Wochenschr 1985;110:1803–1807.
24 van Dam AP, Kuiper H, Vos K, *et al.* Different genospecies of *Borrelia burgdorferi* are associated with distinct clinical manifestations of Lyme borreliosis. Clin Infect Diseases 1993;17:708–717.

25 Mantienne C, Albucher JF, Catalaa I, *et al.* MRI in Lyme disease of the spinal cord. Neuroradiology 2001;43:485–488.
26 Good CD, Jager HR. Contrast enhancement of the cerebrospinal fluid on MRI in two cases of spirochaetal meningitis. Neuroradiology 2000;42:448–450.
27 Engelter S, Lyrer P, Radu EW, *et al.* Acute infectious disorders of the spinal cord and its roots with gadolinium-DTPA enhancement in magnetic resonance imaging. J Neurol 1996;243:191–195.
28 Kruger H, Heim E, Schuknecht B, *et al.* Acute and chronic neuroborreliosis with and without CNS involvement: a clinical, MRI, and HLA study of 27 cases. [see comments]. J Neurol 1991;238:271–280.
29 Kohler J, Kern U, Kasper J, *et al.* Chronic central nervous system involvement in Lyme borreliosis. Neurology 1988;38:863–867.
30 Cadavid D, Thomas DD, Crawley R, *et al.* Variability of a bacterial surface protein and disease expression in a possible mouse model of systemic Lyme borreliosis. J Exp Med 1994;179:631–642.
31 Cadavid D, Bai Y, Dail D, *et al.* Infection and inflammation in skeletal muscle from non-human primates infected with different genospecies of the Lyme disease spirochete *Borrelia burgdorferi*. Infect Immun 2003;71:7087–7098.
32 Roberts ED, Bohm Jr RP, Cogswell FB, *et al.* Chronic lyme disease in the rhesus monkey [see comments]. Lab Invest 1995;72:146–160.
33 van den Berg RH, Faber-Krol MC, Sim RB, *et al.* The first subcomponent of complement, C1q, triggers the production of IL-8, IL-6, and monocyte chemoattractant peptide-1 by human umbilical vein endothelial cells. J Immunol 1998;161:6924–6930.
34 Grewal RP, Morgan TE, Finch CE. C1qB and clusterin mRNA increase in association with neurodegeneration in sporadic amyotrophic lateral sclerosis. Neurosci Lett 1999;271:65–67.
35 Liu L, Tornqvist E, Mattsson P, *et al.* Complement and clusterin in the spinal cord dorsal horn and gracile nucleus following sciatic nerve injury in the adult rat. Neuroscience 1995;68:167–179.
36 Klein MA, Kaeser PS, Schwarz P, *et al.* Complement facilitates early prion pathogenesis. Nat Med 2001;7:488–492.
37 Dietzschold B, Schwaeble W, Schafer MK, *et al.* Expression of C1q, a subcomponent of the rat complement system, is dramatically enhanced in brains of rats with either Borna disease or experimental allergic encephalomyelitis. J Neurol Sci 1995;130:11–16.
38 Kalish RA, Kaplan RF, Taylor E, *et al.* Evaluation of study patients with Lyme disease, 10–20-year follow-up. J Infect Dis 2001;183:453–460.
39 Klempner MS. Controlled trials of antibiotic treatment in patients with post-treatment chronic Lyme disease. Vector Borne Zoonotic Dis 2002;2: 255–263.