

Cardiac involvement in non-human primates infected with the Lyme disease spirochete *Borrelia burgdorferi*

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To investigate cardiac involvement in the non-human primate (NHP) model of Lyme disease, we inoculated 39 adult *Macaca mulatta* with *Borrelia burgdorferi sensu stricto* strains N40 (BbN40) by needle ($N=22$, 14 immunocompetent (IC), seven permanently immunosuppressed (IS), and four transiently immunosuppressed (TISP)) or by tick-bite ($N=4$, all TISP) or strain 297 (Bb297) by needle ($N=2$ IS), or with *B. garinii* strains Pbi ($N=4$, 2 TISP and 2 IS), 793 ($N=2$, TISP) or Pli ($N=2$, TISP). Five uninfected NHPs were used as controls. Infection and inflammation was studied in the hearts and the aorta removed at necropsy 2–32 months after inoculation by (1) H&E and trichrome-staining; (2) immunohistochemistry and digital image analysis; (3) Western blot densitometry; and (4) TaqMan RT-PCR. All NHPs inoculated with BbN40 became infected and showed carditis at necropsy. The predominant cells were T cells, plasma cells, and macrophages. There was increased IgG and IgM in the heart independent of immunosuppression. The B-cell chemokine BLC was significantly increased in IS-NHPs. There was increased deposition of the complement membrane attack complex (MAC) in TISP and IS-NHPs. The spirochetal load was very high in all BbN40-inoculated IS-NHPs but minimal if any in IC or TISP NHPs. Double-immunostaining revealed that many spirochetes in the heart of BbN40-IS NHPs had MAC on their membranes. We conclude that carditis in NHPs infected with *B. burgdorferi* is frequent and can persist for years but is mild unless they are immunosuppressed.

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Lyme borreliosis is a systemic disease caused by infection with the spirochete *Borrelia burgdorferi*.¹ It is currently the most common arthropod-borne disease in the United States, where thousands of cases are reported to the Center for Disease Control every year.² At least three genospecies pathogenic to humans have been characterized: *B. burgdorferi sensu stricto*, *B. afzelii*, and *B. garinii*. Only *B. burgdorferi sensu stricto* is endemic in North America, while all three genospecies are endemic in Europe. The organs most often affected are the skin, the joints, the heart, and the central and peripheral nervous system.

Cardiac manifestations of *B. burgdorferi* infection occur in up to 8% of patients.^{1,3} Clinically, Lyme carditis is typically characterized by varying degrees of intermittent atrioventricular block occurring within weeks of infection with *B. burgdorferi*, a median of 21 days from the onset of erythema migrans. Temporary cardiac pacing may be required in up to one-third of cases. Myocarditis and/or pericarditis may occur and also mild left ventricular dysfunction that rarely can be fatal.¹ Cardiomyopathy has been associated with *B. burgdorferi* infection in Europe but not in the United States.⁴

Our laboratory has been studying the pathogenesis of Lyme borreliosis using non-human primates (NHPs) for several years.^{5–10} During infection of immunosuppressed NHPs inoculated with the *sensu stricto* strain N40 of *B. burgdorferi* (BbN40), we found that the heart had the most severe injury of all tissues examined and one of the highest spirochetal loads.⁶ The goal of the present study was to

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characterize the full spectrum of Lyme carditis in the NHP model using different strains of *B. burgdorferi* and degrees of immunosuppression during both short- and long-term infection and after syringe or tick-inoculation. The results showed that cardiac inflammation was a consistent finding in all NHPs inoculated with BbN40 but was mild unless the animals were immunosuppressed.

Materials 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,¹¹ Bb297 is a CSF isolate from a patient in Connecticut.¹² BgPbi and BgPli are CSF isolates from Europe,¹³ and Bg793 is a tick isolate from Europe.

Animals Inoculation and Necropsy

A total of 39 adult *Macaca mulatta* were inoculated intradermally with different strains of *B. burgdorferi sensu stricto* or *garinii* as follows: BbN40 by needle ($N=22$) or by tick-bite ($N=4$); Bb297 by needle ($N=2$); or BgPbi ($N=4$), Bg793 ($N=2$) or BgPli ($N=4$) all by needle. The methods for tick or needle inoculation and for immunosuppression have been reported before⁵ as well as results for infection of all the immunosuppressed animals inoculated with BbN40, Bb297, and *garinii* strains. The hearts from five NHPs that were uninfected were used as negative controls.

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. Paraffin sections were cut at $5\mu\text{m}$ and cryomatrix sections at $8\mu\text{m}$. Inflammation was assessed by examination of hematoxylin and eosin (H&E) staining. To compare the severity of inflammation in heart, H&E-stained sections were graded by a masked examiner for the severity of inflammation per $\times 40$ microscopic field as follows: absent inflammation = 0; 1–3 foci = 1+ (minimal); 4–10 foci = 2+ (mild); 11–20 foci = 3+ (moderate); and more than 20 foci: 4+ (severe).

Enzyme-Linked Immunosorbent Assay and Immunoblot

Serum enzyme-linked immunosorbent assay (ELISA) and Western blots were performed as described.^{5,7,14} The strain used for preparation of most Western

blots was *B. burgdorferi sensu stricto* strain CB, an isolate from an erythema migrans lesion from Valhalla, NY. ELISA and immunoblots were repeated for the *garinii*-inoculated NHPs using antigens from *B. garinii* strains.

Immunohistochemistry and Image Analysis

Immunohistochemistry and digital image analysis were performed as previously described.^{6,7} Antigen retrieval by microwave heating (Dako's target retrieval solution, Code No. S1699) or protease digestion (P-6911, Sigma Protease) was used with formalin-fixed tissues. Rabbit polyclonal antibody antihuman IgG (Dako's A0423), IgM (Dako's A0425), C1q (Dako's A0136), and CD3 (T cell marker, Dako's A0452), mouse monoclonal antibody anti-P63 (plasma cell marker, Dako's M7077), Ham56 (monocyte/macrophage marker, Dako's M0632), C9 neopeptide (MAC) (Dako's M0777), and goat anti-human BLC/BCA(R&D AF801) were used as primary antibody. Recombinant human BLC/BCA peptide (R&D, 801-CX) was used for blocking assays of the anti-BLC antibody to confirm its specificity. Primary antibody for detection of *B. burgdorferi* was hyperimmune serum from a rabbit persistently infected with *B. burgdorferi* strain N40.¹⁵ For negative controls, duplicate sections on each glass slide were incubated with affinity-purified nonspecific antibody (Sigma) matched for concentration, species, and isotype. Spleen or lymph node tissues were used as positive controls for markers of inflammation.

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 (YB) took 4–6 digital images at $\times 40$, $\times 100$, or $\times 200$ -magnification. The mean (s.d.) sum area (in square microns) and sum optical density (in arbitrary units) were determined and compared between groups. The intensity of immunostaining for some markers (CD3, P63, and C1q) was compared manually by semiquantitation of the intensity of the staining as absent (0), mild (1), moderate (2), or severe (3) by a masked examiner.

Immunofluorescence Staining

C5b-9 (MAC) and *B. burgdorferi* double immunofluorescence staining was performed with fluorescein isothiocyanate (FITC)-conjugated (Sigma, F0382) anti-rabbit and tetramethyl rhodamine isothiocyanate (Tritc)-conjugated anti-mouse goat polyclonal antibody (Sigma, T6528) at 1/250 dilution. Adobe Photoshop V7.0 software was used to merge single color images.

PCR

Total RNA was extracted with TRIzol reagent (Life technologies) from 100 mg NHP tissue blocks. The

reverse transcription (RT) was performed in 20- μ l reaction volumes. Taqman RT-PCR for the 16S rRNA of *Borrelia* spp. was performed as described.^{5,7} PCR-ELISA for the OspA or OspB *B. burgdorferi* genes was performed as described.⁶ All assays were run in triplicate.

Immuno Dot-Blot

Immuno dot-blot was performed as described.⁷ Protein concentration was determined in the supernatant by the BCA protein assay (Pierce). Dot-blots were prepared by spotting 0.02–0.2 μ g in duplicate from each protein extract to polyvinylidene difluoride membranes (Millipore). The primary antibodies were rabbit polyclonal anti human IgG (Dako) or IgM at a 1:5000 dilution. The secondary antibody was alkaline-phosphatase-conjugated goat anti-rabbit IgG (Sigma). After incubation in fluorescence substrate ECF (Amersham's RPN5785) for 5 min, the membranes were scanned with the Typhoon 8600 (Amersham Pharmacia Biotech Inc.). Results were analyzed by densitometry using Image-Quant Software and expressed as mean (s.d.).

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 the SPSS software version 10. The *P*-values lower than 0.05 were considered significant. For immuno dot-blot, results were compared for significance by Student's *t*-test.

Results

Animal Infections

A total of 39 adult NHPs inoculated with different strains of *Borrelia burgdorferi* were used for these studies. The results of infection of all animals that were immunosuppressed have been published.⁵ Table 1 lists the results of infection of all immunocompetent NHPs inoculated intradermally with *B. burgdorferi* strain N40. None of the NHPs developed erythema migrans and no signs of neurological disease were apparent to care takers. Serial examination of cerebrospinal fluid (CSF) failed to show any evidence of CSF-leukocytosis. Viable spirochetes were identified by tissue culture at necropsy only in immunosuppressed animals.

Antibody Response

ELISA on necropsy sera showed that all immunocompetent NHPs inoculated with BbN40 developed specific antibody (Table 1). In contrast, only one out of two NHPs inoculated with Bb297 and four out of eight NHPs inoculated with *B. garinii* strains had

Table 1 Infection in immunocompetent NHPs examined by necropsy 2–32 months after intradermal inoculation with *B. burgdorferi sensu stricto* strain N40

NHP ID	Necropsy (months)	α BbELISA ^a	α BbIgM WB	α BbIgG WB
TO26	32	+	–	+
TO29	31	+	–	+
PAX40	20	+	–	+
TO32	8	+	–	+
E520	6.5	+	–	+
M14	6	+	+	+
M15	6	+	+	+
E518	5	+	–	+
DES9	4	NA	NA	NA
E78	4	NA	NA	NA
U368	4	NA	NA	NA
23Z	4	+	+	+
E680	4	+	+	+
1616	2.5	+	–	+

^aAntibody measured at necropsy by ELISA or Western blot (WB) with homologous sonicate.

detectable anti *B. burgdorferi* antibody by ELISA and at low titers.⁵ The immunoblot for all inoculated NHPs have been reported.^{5,16,17} Table 1 also summarizes the immunoblot results for the BbN40-inoculated immunocompetent NHPs: all that were tested had positive Lyme IgG WB at necropsy and none had positive IgM WB when examined later than 6 months after inoculation. All WB from garinii-inoculated NHPs were negative even when tested with homologous sonicates,⁵ an indication that NHPs are resistant to syringe inoculation of garinii strains of Lyme disease borrelias.

Inflammation in the Heart

The aorta and the atrium, ventricle and apex of the hearts from all NHPs were examined for the presence of inflammation (carditis) by light microscopy of H&E-stained paraffin and frozen sections (Figure 1a). The results showed inflammation in at least one cardiac tissue block from all but four of the 39 inoculated NHPs. No carditis was observed in any of the five uninfected controls. To compare the frequency and severity of inflammation, we calculated a mean sum inflammatory score (see Materials and methods) (Table 2). The highest mean sum scores were 0.31, 0.30, and 0.28 for the BbN40-inoculated TISP-tick, IS, and TISP-needle NHPs, respectively. The mean sum inflammatory score was also increased in the IC-short-term and long-term groups (0.20 and 0.26) and in the Bg793 garinii group (0.28) (not shown). Trichrome staining of the ventricles of BbN40-inoculated NHPs showed most had increased collagen deposition compared with uninfected controls (Figure 1b). The mean (s.d.) sum density score for collagen per \times 40 microscopic field was 2638 (2231), 12 327 (11 351), 13 762 (8176), and

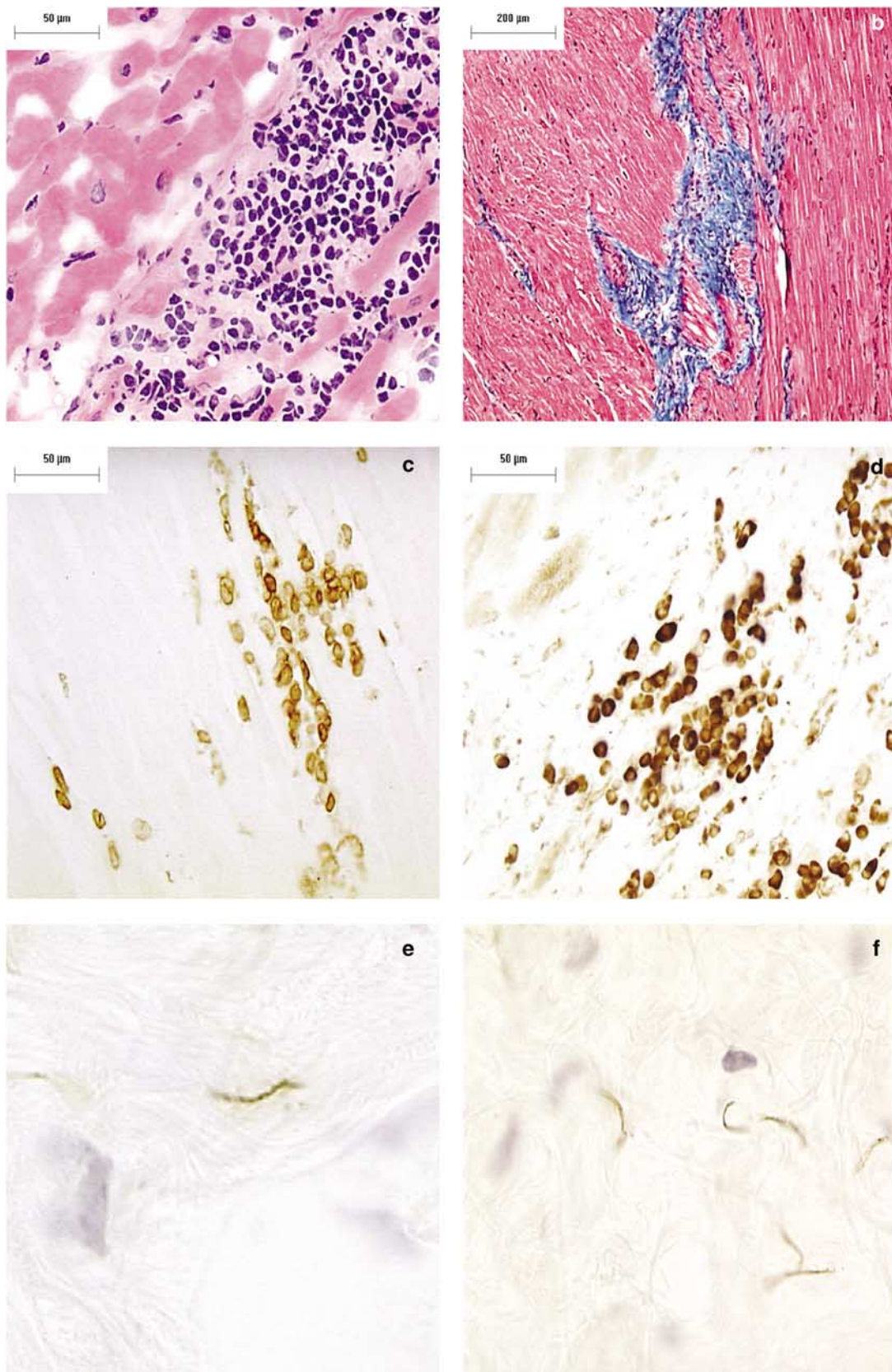


Figure 1 Inflammation in NHP M15 heart inoculated with BbN40 (panel a, H&E $\times 400$); Trichrome staining in 372 IS-NHPs heart (panel b, $\times 100$); T cells in IS-NHP 372 heart (panel c, CD3 immunostaining $\times 400$); plasma cells in IS-NHP 1614 heart (panel d, P63 immunostaining $\times 400$); *B. burgdorferi* in the aorta (panel e) and atrium (panel f) of IS NHP 571 inoculated with Bb297 (*Borrelia* immunostaining $\times 1000$ in panel a and $\times 400$ in panel f).

Table 2 Inflammation by H&E in biopsy heart from immunocompetent (IC) or transiently (TISP) or permanently (IS) immunosuppressed NHPs inoculated with *B. burgdorferi* N40 by needle or by tick-bite compared with uninfected controls

NHP ID	Strain	Inoculation	Immunosuppression	H&E				Mean sum score	
				Aorta	Atrium	Ventricle	Apex	NHP	Group
TO26	BbN40	Needle	IC-long term	1	1	NA	NA	2/8 ^a	0.26 ^b
TO29			IC	1	1	1	NA	3/12	
PAX40			IC	1	1	1	NA	3/12	
TO32			IC	1	2	1	2	6/16	
E520			IC	1	0	1	NA	2/12	
E518	BbN40	Needle	IC-short term	2	0	3	0	5/16	0.20
DES9			IC	1	1	0	1	3/16	
E78			IC	2	2	2	2	8/16	
U368			IC	0	0	0	0	0/16	
M14			IC	0	3	0	1	4/16	
M15			IC	0	0	1	3	4/16	
23Z			IC	1	0	0	0	1/16	
E680			IC	1	1	0	0	2/16	
1616			IC	3	0	0	0	3/16	
652			BbN40	Needle	IS	0	1	2	
1538	IS	3			1	3	0	7/16	
Z1	IS	3			0	0	0	3/16	
PAX219	IS	2			3	1	3	9/16	
1614	IS	2			3	0	0	5/16	
30794	IS	0			0	0	2	2/16	
372	IS	0	0	2	1	3/16			
099	BbN40	Needle	TISP	0	2	3	2	7/16	0.28
177			TISP	0	2	1	0	3/16	
199			TISP	0	2	1	0	3/16	
383			TISP	0	2	2	1	5/16	
154	BbN40	Tick	TISP	0	1	1	0	2/16	0.31
192			TISP	0	2	3	1	6/16	
211			TISP	0	3	3	3	9/16	
242			TISP	0	1	1	1	3/16	
M5	None	None	IC	0	0	0	0	0/16	0
M6			IC	0	0	0	0	0/16	
316			IC	NA	NA	0	NA	0/4	
650			IC	NA	NA	0	NA	0/4	
054			IC	NA	NA	0	NA	0/4	

^aNumber of points for inflammation over number of maximum point possible (range 0–4, see Materials and methods for details).

^bSum of all points for inflammation severity in the group divided by number of maximum points possible.

NA = Not available.

21 246 (24 807) for uninfected controls, IS, TISP-tick, and IC-short term NHPs, respectively (*P*-value <0.01 for all groups compared with uninfected controls).

Light microscopic examination indicated that the predominant inflammatory cells were mononuclear cells, many with morphological features of plasma cells. To further characterize the inflammatory infiltrate we did immunostaining for T cells (CD3, Figure 1c), plasma cells (P63, Figure 1d), and macrophages (Ham56) and compared them manually (for T cells and plasma cells, Table 3) or by digital image analysis (for macrophages, Figure 2). The results showed that there were more T cells and plasma cells in the IS group, followed by the short-term-IC group. The extent of macrophage infiltration

was higher in short-term-IC, IS, TISP-tick, and TISP-needle NHPs than in uninfected controls or the long-term-IC group (*P*-value compared with uninfected controls was <0.05 for short-term-IC and <0.01 for TISP-needle and TISP-tick).

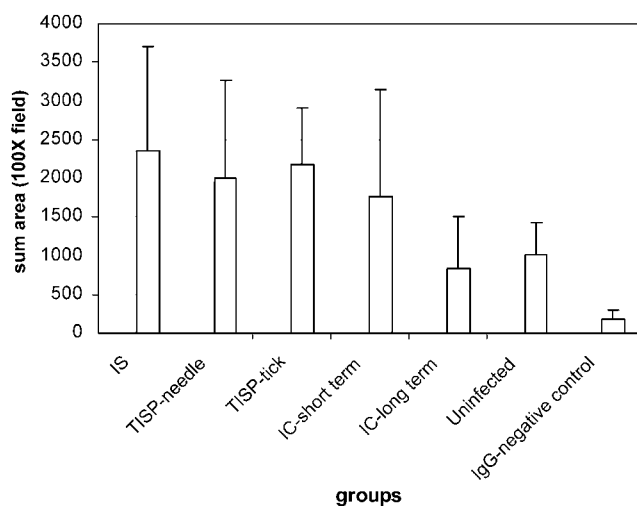
Spirochetal Localization and Numbers

To investigate the localization of spirochetes in the heart, we examined tissue sections immunostained with anti-*B. burgdorferi* specific antibody. Spirochetes were found in the aorta and the heart from BbN40 or Bb297 inoculated IS-NHPs (Figure 1, panels e and f). Some areas had very large numbers of spirochetes, as many as 5–10 per × 400

Table 3 Semiquantitation of CD3, P63 and C1q protein expression in the heart of NHPs inoculated with *B. burgdorferi sensu stricto* strain N40

NHP ID	Inoculation	Immunosuppression	CD3	P63	C1q
E518	Needle	IC-short term	1 ^a	1	0–1
23Z		IC	1	1	0–1
E680		IC	1	1–2	2
1616		IC	1–2	1	0
652	Needle	IS	2	1	0
1538		IS	2–3	2–3	0
Z1		IS	2	2–3	1
PAX219		IS	2–3	2–3	2
1614		IS	2	2	0
30794		IS	2	2	1
372		IS	2	2	1
099	Needle	TISP	1	1	0
177		TISP	2	1	0
199		TISP	2	1	0
383		TISP	2	1	0
154	Tick	TISP	2	1	0
192		TISP	2	1	0
211		TISP	2	2	0
242		TISP	2	0–1	0
M5	None	IC	0	0	0–1
M6			0	0	0

^aThe intensity of the staining as absent (0), mild (1), moderate (2), or frequent (3).

**Figure 2** Digital image analysis of immunostaining with antibody anti-Ham 56 (macrophage marker) in the heart from different groups of NHPs inoculated with *B. burgdorferi* by needle or tick-bite.

microscopic field (Figure 1f). Their localization was predominantly in connective tissue in the aorta and the heart atrium and ventricle (endocardium, pericardium, and epicardium). In no case, they appeared to be intracellular in macrophages or cardiac myocytes.

To investigate the presence of *B. burgdorferi* in tissues at necropsy, we used OspA or OspB PCR-ELISA or Borrelia 16S rRNA TaqMan RT-PCR (Table 4). The results showed that in all tissues examined from IC or TISP NHPs the signal was either negative or only weakly positive, with inconsistent results when multiple areas from the same heart were examined (not shown). In contrast, the TaqMan RT-PCR detected large numbers of spirochetes in the heart of BbN40-inoculated IS-NHPs (Table 5).

Antibody Deposition

Plasma cells were common in the heart from all BbN40-inoculated NHPs. Since the production of immunoglobulin is the primary function of plasma cells, we next looked for the presence of antibody in hearts from BbN40-inoculated NHPs. Light microscopic examination revealed extensive deposition of IgG and IgM in the membranes of cardiac myocytes and blood vessels and in the connective tissue throughout the heart and the aorta (not shown). Digital image analysis showed significantly increased deposition of IgG in all BbN40-inoculated NHPs compared with uninfected controls ($P < 0.001$) (Figure 3a). There were also significant differences in IgM deposition (Figure 3b). IS and to a lesser extent TISP-NHPs but not the short-term-IC group had significantly increased IgM deposition compared with uninfected controls ($P < 0.01$).

To confirm if the hearts from IS-NHPs had higher antibody deposition than the TISP groups, we did dot-blot densitometry in whole-protein extracts from ventricles (Table 6). The results confirmed that there was significantly more IgM in IS than in both TISP NHP groups. It also revealed higher IgM in the TISP-needle compared with the TISP-tick and higher IgG in the TISP-needle than in the other two groups.

Complement Deposition

Immunohistochemistry showed deposition of the first component of the complement cascade (C1q) in the heart from some inoculated NHPs (Figure 4a). The localization was predominantly membrane bound, perivascular, and in collagenous areas. To investigate whether there were differences in the deposition of C1q, we did manual semiquantitation (Table 3). Only some of the short-term-IC and IS NHPs inoculated with BbN40 showed increased C1q deposition by light microscopy. However, dot-blot densitometry showed that the amount of C1q was significantly higher in IS than in any of the two groups of TISP-NHPs (Table 6).

To investigate whether antibody and C1q deposition was associated with deposition of the membrane attack complex (MAC/C5b-9), we did immunohistochemistry with an anti-human MAC primary antibody. The results showed the presence

Table 4 Infection by PCR in heart from immunocompetent (IC) or transiently (TISP) or permanently (IS) immunosuppressed NHPs inoculated with *B. burgdorferi* N40 by needle or by tick-bite

NHP ID	Strain	Inoculation	Immunosuppression	16s RT-PCR or PCR-ELISA (<i>OspA/B</i>)			
				Aorta	Atrium	Ventricle	Apex
TO26	BbN40	Needle	IC-long term	–	–	nd	–
TO29			IC	nd	nd	nd	nd
PAX40			IC	nd	nd	nd	nd
TO32			IC	–	nd	nd	–
E520			IC	+	+/-	nd	–
E518	BbN40	Needle	IC-short term	nd	–	nd	nd
M14			IC	–	–	–	–
M15			IC	–	–	–	–
23Z			IC	nd	+	nd	nd
E680			IC	–	–	nd	–
1616			IC	nd	–	nd	nd
652			BbN40	Needle	IS	+	+
1538	IS	nd			+	nd	+
Z1	IS	nd			+	nd	+
PAX219	IS	nd			+	nd	+
1614	IS	nd			+	nd	+
30794	IS	+			+	+	+
372	IS	+			+	+	+
099	BbN40	Needle	TISP	nd	nd	+	nd
177			TISP	nd	nd	+	+
199			TISP	+	nd	nd	nd
383			TISP	+	nd	nd	nd
154	BbN40	Tick	TISP	+	+	nd	nd
192			TISP	+	+	nd	nd
211			TISP	+	+	+	+
242			TISP	+	nd	nd	nd

nd = not determined.

Table 5 Spirochetal load^a by Taqman RT-PCR in the heart and aorta of IS NHPs inoculated with *B. burgdorferi sensu stricto* strain N40

NHP ID	Aorta	Atrium	Ventricle	Apex
652	16 100	2750	83	2960
30794	11 042	426 095	468 452	836 041
372	805 619	196 232	164 402	413 009

^aNumber of spirochetes per 100 mg tissue.

of MAC not only in the membranes from cardiac myocytes (Figure 4c) but also in spirochetes (Figure 4e). Detailed examination of the MAC-stained spirochetes suggested that many appeared intact while others appeared mildly damaged or degraded. To investigate whether MAC deposition increased in the heart as a result of infection, we compared MAC deposition by digital image analysis. The results (Figure 5) showed significantly increased MAC in all groups of inoculated NHPs compared with uninfected controls. The three highest values were for TISP-needle, TISP-tick and IS-BbN40-inoculated NHPs. Mild but significantly increased MAC

deposition was also found in the garinii NHPs compared with uninfected controls.

To confirm whether MAC was being deposited in spirochetes as suggested by immunohistochemistry, we did double immunofluorescence staining. The results showed colocalization of MAC and *Borrelia* proteins on spirochetes (Figure 4b, d, f). Examination of heart sections from IS-NHPs revealed that there were both MAC-positive and MAC-negative spirochetes throughout. These results showed that in steroid-treated NHPs heavily infected with BbN40 MAC binds to but does not kill spirochetes.

B-Lymphocyte Chemoattractant (BLC)

The previous experiments demonstrated extensive accumulation of plasma cells and antibody in the hearts of NHPs with Lyme carditis. To investigate whether specific B-cell chemokines were being produced in the hearts as a result of the infection that could be responsible for plasma cell infiltration, we looked for the presence of the B-cell chemokine BLC/CXCL13. Digital image analysis of formalin-fixed immunostained sections showed significant accumulation of BLC/CXCL13 mainly in IS and

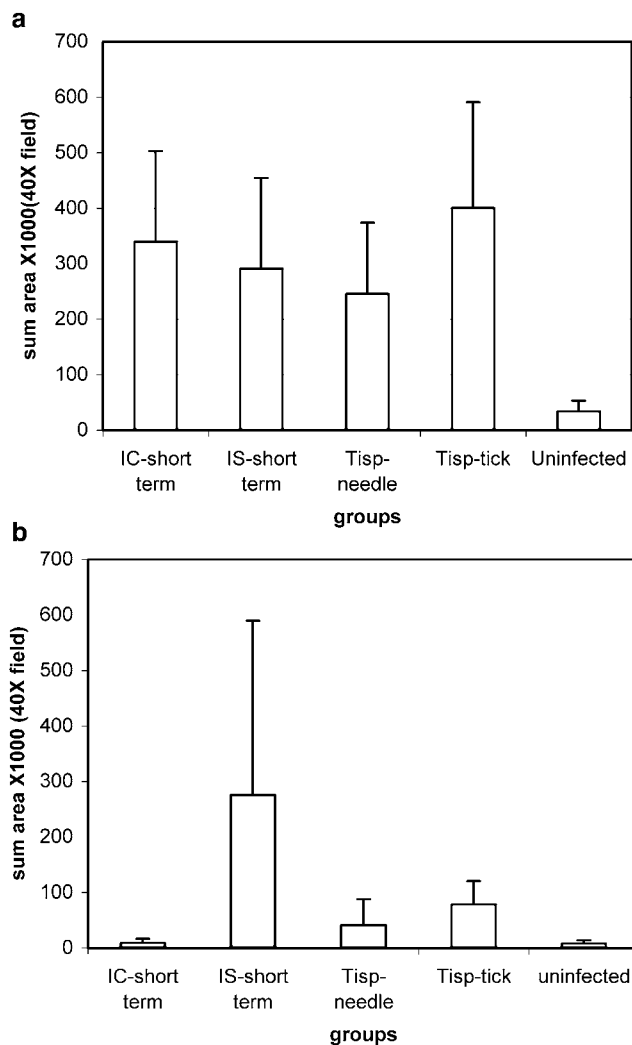


Figure 3 Digital image analysis of immunostaining with antibody anti-IgG (a) or anti-IgM (b) on heart from different groups of NHPs inoculated with *B. burgdorferi* by needle or tick-bite.

Table 6 IgG, IgM and C1q protein levels by quantitative dot blot in the ventricle of NHPs inoculated with BbN40

Group	IgG	IgM	C1q
IS	2.92 ± 0.24 ^a	48.42 ± 3.49 ^b	1.16 ± 0.02 ^b
TISP-needle	3.66 ± 0.25 ^d	43.26 ± 4.19 ^c	0.57 ± 0.04 ^c
TISP-tick	3.01 ± 0.14	36.65 ± 6.34	0.52 ± 0.04

^aMean (SD) density per 0.02 μg (IgG), 0.2 μg (IgM, C1q) of protein extract (× 1/10⁶).

^bThe *P*-value was <0.001 for group IS vs TISP.

^cThe *P*-value was <0.001 for group TISP-needle vs TISP-tick.

^dThe *P*-value was <0.001 for group TISP-needle vs IS.

to a lesser extent in IC-short-term and TISP-NHPs (Figure 6). To make sure the signal from the anti-BLC/CXCL13 antibody was specific, we repeated the immunostaining with and without blocking with

BLC peptide. The results (not shown) confirmed the anti-BLC antibody was specific.

Discussion

This manuscript presents the first comprehensive investigation of cardiac involvement in Lyme borreliosis in primates. The main findings of the study were as follows: (1) carditis is very common in NHPs infected with *B. burgdorferi* but is mild unless the animals are immunosuppressed. (2) The spirochetal load in the heart is very high in NHPs necropsied while immunosuppressed but decreased to minimal or undetectable in all NHPs necropsied while immunocompetent. (3) The cellular inflammatory response to the infection was characterized by multifocal collections of T cells, plasma cells, and macrophages. (4) Infection resulted in increased deposition of IgG and IgM in the heart. (5) Expression of the B-cell chemokine BLC was increased accordingly to the spirochetal load. (6) Increased deposition of the complement membrane attack complex (MAC) was found in the heart from TISP and IS-NHPs, and a significant percentage of the spirochetes in the heart had MAC on their membranes.

A previous report from our group described significant cardiac inflammation and tissue injury in the heart of immunosuppressed NHPs infected with the BbN40 strain.⁶ We had also seen extensive cardiac injury in the heart of mice with severe combined immunodeficiency infected with *B. turicatae*.¹⁸ In humans with Lyme disease, carditis is found in up to 25%, but only rarely pathology specimens are available for examination.¹⁹ In NHPs we found that all but one inoculated with the BbN40 strain had evidence of carditis at necropsy, including some that had been inoculated years before. However, carditis was overall mild. The pattern of inflammation was multifocal and patchy, although occasional large lesions were found (color Figure 1a).

All groups of BbN40-inoculated NHPs had similar macrophage infiltration with the exception of the long-term-IC group (Figure 2). The finding of increased numbers of T cells, plasma cells (Table 3, Figure 1c and d), and macrophages (Figure 2) in the heart is consistent with previous observations in small animal models of Lyme carditis.^{20–22} Plasma cells were more abundant in IS-NHPs (Table 3), which was also the group with the highest expression of BLC (Figure 6) and the highest tissue deposition of IgM (Figure 4, panel b). This suggests that one of the consequences of persistent *B. burgdorferi* infection of the heart is upregulation of the B-cell chemokine BLC leading to infiltration by plasma cells and production and deposition of large amounts of IgM. The specificity of the IgM antibody deposited in heart tissue has not been determined. It is also unknown why this IgM antibody is unable to

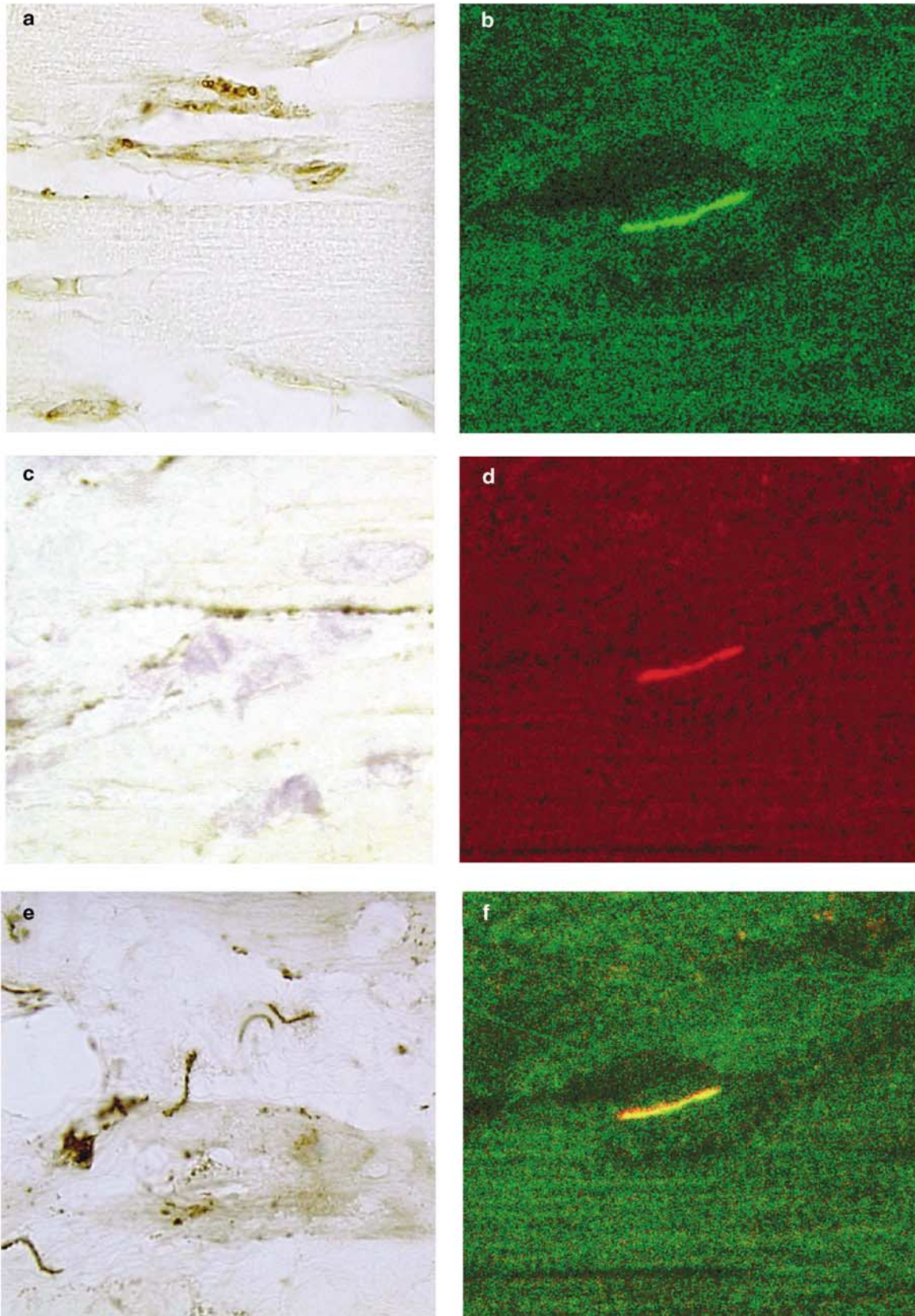


Figure 4 C1q (panel a) and MAC (panels c and e) immunostaining in the heart of IS NHP 31372. MAC & *Borrelia* double immunofluorescent staining with FITC conjugate anti BbN40 antibody (panel b, green color), TRITC conjugated anti MAC antibody (panel d, red color) and merged images from panels b and d (panel f, yellow color).

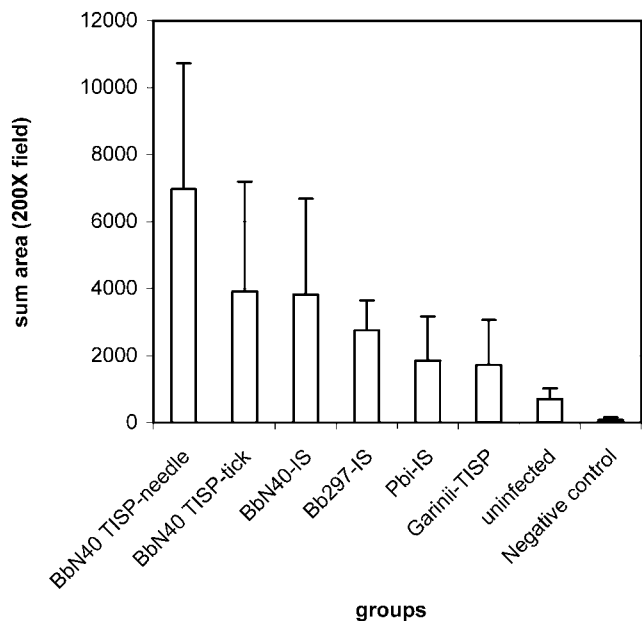


Figure 5 Digital image analysis of immunostaining with antibody anti-MAC (C9 neopeptide) on heart from different groups of NHPs inoculated with different genospecies of *B. burgdorferi*.

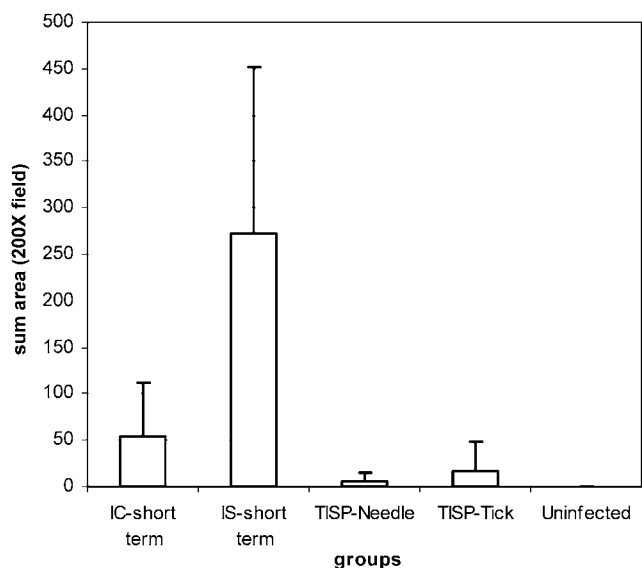


Figure 6 Digital image analysis of immunostaining with antibody anti-BLC on heart from different groups of NHPs inoculated with BbN40.

kill the spirochetes, as shown by the very high spirochetal load present in the heart of IS-NHPs (Table 5).

BLC (also called BCA-1 or CXCL13) is a chemokine thought to be especially selective for B-cells. BLC is considered a homing chemokine and has been implicated in the trafficking of lymphocytes and dendritic cells in lymphoid organs, and is critical for lymphoid neogenesis²³ and for establishment of lymphoid follicle-like areas in chronically inflamed tissues such as salivary glands in Sjogren's

syndrome²⁴ or joins in rheumatoid arthritis.²⁵ In a previous study, we found significantly increased levels of BLC mRNA in skeletal muscle from TISP-NHPs inoculated with BbN40 compared with controls that were uninfected or inoculated but not infected.²⁶

As expected, a dramatic effect of the immunosuppression was an inability to fight the infection. One reason why IS-NHPs failed to control the infection was the lower levels of circulating specific antibody, as previously reported.^{5,7} Consistent with this are the results of the dot-blot analysis (Table 6) that showed higher total IgG in the heart of TISP compared with IS-NHPs. Although digital image analysis showed similar levels of IgG, this is a less sensitive technique than the dot blot. In contrast to IgG, IS-NHPs had much higher IgM as shown both by dot blot and digital image analysis. As discussed above, it is unclear why this IgM antibody failed to control the infection.

Another possibility for the higher spirochetal load in steroid-treated animals is impaired complement activation that is required for efficient spirochetal killing. In the absence of specific antibody *B. burgdorferi* is resistant to the bactericidal activity of complement.²⁷ Bactericidal antibody appears necessary for the effective formation of MAC.²⁷ Most pathogenic microorganisms, and in particular those that circulate in the blood stream like spirochetes, develop a wide range of strategies to elude antibody and complement killing. We found that a large percentage of spirochetes in the heart of IS-NHPs had MAC on their membranes (color Figure 4e), including many that appear morphologically intact. The reason why spirochetes appear to survive MAC deposition in IS-NHPs is not known. One possibility is disabling the correct assembly of MAC. MAC is an ultrastructurally heterogeneous complex that induces the formation of membrane channels of different sizes.²⁸ Patarakul *et al*²⁹ found similar level of MAC on the membrane of a complement-resistant *B. burgdorferi* (WT297) and a complement-sensitive mutant (MUT297). Although both had polymerization of C9 and MAC diffusely distributed and tightly bound on the outer membrane, protease treatment rendered WT297 but not MUT297 susceptible to serum killing. Proteins of 20, 30, and 66 kDa were found in the membrane of WT297 but not in MUT297 that may be responsible for complement resistance.²⁹ Two type of proteins that may be involved in complement resistance in *B. burgdorferi* have been reported. One of them is OspE/Erp proteins that bind factor H.^{30,31} More recently, a CD59-like molecule that inhibits the assembly of MAC was described on the outer membrane of *B. burgdorferi*.³²

We found deposition of MAC in the heart of IS-NHPs not only on spirochetes but also on the membranes of cardiac myocytes. In Chagas cardiomyopathy, another form of infectious carditis caused by parasite *Trypanosoma cruzi*, MAC was found in

the sarcolemma of 38% of cases compared with 0% of controls.³³ MAC deposition is also a feature of damaged myocytes in myocardial infarction.³⁴ We propose that in Lyme carditis complement activation in response to the spirochetal infection leads to MAC deposition in cell membranes of cardiac myositis with secondary fiber degeneration and fibrosis.

The true prevalence of Lyme carditis in humans is difficult to determine because only few cases have been examined at autopsy. The incidence of symptomatic Lyme carditis has been estimated to be 4–10% in adults. However, the incidence of abnormal ECG findings in asymptomatic patients with probable or definite Lyme borreliosis is higher, 29% in one study in children.³⁵ The clinical course of Lyme carditis is usually benign with most patients recovering completely. In rare instances, death has been reported.^{19,36} The cardinal manifestation is conduction system disease, which generally is self-limited. Heart block occurs usually at the level of the atrioventricular node but often is unresponsive to atropine sulfate. Temporary pacing may be necessary in more than 30% of patients, but permanent heart block rarely develops. Myocardial and pericardial involvement can occur but generally is mild and self-limited.³⁷ Cardiomyopathy has been associated with *B. burgdorferi* infection in Europe but not in the United States.⁴ No treatment has been shown clearly to attenuate or prevent the development of Lyme carditis, but mild carditis generally is treated with oral antibiotics and severe carditis with intravenous antibiotics.³⁷

Studies in mouse models of Lyme borreliosis showed that Lyme carditis is very frequent but until now the true incidence in primates was not known. Histopathological examination of mice with severe combined (*scid*)³⁸ or other (NIH-3)³⁹ immunodeficiency inoculated with *B. burgdorferi* show high prevalence of severe carditis. Lyme carditis is also prominent in immunocompetent mice^{20,21} and is worse in IL-4-deficient mice.²² In C3H mice, spirochetes have been found in the heart as early as 6 days after inoculation and all mice of the C3H and C57BL/6 haplotypes had infected hearts.²⁰ The spirochetes had a predilection for connective tissue in the heart base. Carditis was first detectable on day 10, peaked on day 15, and resolved except for persistence of periaortic lymphoplasmacytic infiltrates in all mice. The C3H mice developed more severe disease than the C57BL/6 mice, and this was associated with earlier appearance, greater numbers, and later clearance of spirochetes in C3H mice.²⁰ A previous study of rhesus macaques inoculated with the JD-1 strain of *B. burgdorferi* found focal myocarditis in three out of six hearts examined at necropsy 6 months later.⁴⁰

In summary, this study revealed Lyme carditis is very common in infected NHPs when examined pathologically. Although severe infection of the heart occurs in the setting of immunosuppression,

the intact immune response of NHPs reduced the infection to minimal or undetectable levels. The failure of steroid-treated NHPs to clear the infection may be the result of impaired killing due to decreased production of specific antibody or failure of MAC assembly on the membranes of spirochetes.

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