0023-6837/00/8007-1043$03.00/0
LABORATORY INVESTIGATION
Vol. 80, No. 7, p. 1043, 2000
Printed in U.S.A.

Localization of *Borrelia Burgdorferi* in the Nervous System and Other Organs in a Nonhuman Primate Model of Lyme Disease

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SUMMARY: Lyme borreliosis is caused by infection with the spirochete *Borrelia burgdorferi*. Nonhuman primates inoculated with the N40 strain of *B. burgdorferi* develop infection of multiple tissues, including the central (CNS) and peripheral nervous system. In immunocompetent nonhuman primates, spirochetes are present in low numbers in tissues. For this reason, it has been difficult to study their localization and changes in expression of surface proteins. To further investigate this, we inoculated four immunosuppressed adult *Macaca mulatta* with 1 million spirochetes of the N40 strain of *B. burgdorferi*, and compared them with three infected immunocompetent animals and two uninfected controls. The brain, spinal cord, peripheral nerves, skeletal muscle, heart, and bladder were obtained at necropsy 4 months later. The spirochetal tissue load was first studied by polymerase chain reaction (PCR)-ELISA of the outer surface protein A (ospA) gene. Immunohistochemistry was used to study the localization and numbers of spirochetes in tissues and the expression of spirochetal proteins and to characterize the inflammatory response. Hematoxylin and eosin and trichrome stains were used to study inflammation and tissue injury. The results showed that the number of spirochetes was significantly higher in immunosuppressed animals. *B. burgdorferi* in the CNS localized to the leptomeninges, nerve roots, and dorsal root ganglia, but not to the parenchyma. Outside of the CNS, *B. burgdorferi* localized to endoneurium and to connective tissues of peripheral nerves, skeletal muscle, heart, aorta, and bladder. Although ospA, ospB, ospC, and flagellin were present at the time of inoculation, only flagellin was expressed by spirochetes in tissues 4 months later. Significant inflammation occurred only in the heart, and only immunosuppressed animals had cardiac fiber degeneration and necrosis. Plasma cells were abundant in inflammatory foci of steroid-treated animals. We concluded that *B. burgdorferi* has a tropism for the meninges in the CNS and for connective tissues elsewhere in the body. (*Lab Invest 2000, 80:1043–1054*).

Lyme borreliosis is a multisystemic disease caused by the spirochete *Borrelia burgdorferi* (Burgdorfer et al, 1982). At least three genospecies have been characterized, *B. burgdorferi* sensu stricto, *B. afzelii*, and *B. garinii*, of which only *B. burgdorferi* sensu stricto is endemic in North America. Lyme borreliosis is currently the most common arthropod-borne disease in the United States, where thousands of cases are reported to the Centers for Disease Control every year (Centers for Disease Control, 1995; 1996). The organs more often affected are the skin, the joints, the heart, and the central (CNS) and peripheral (PNS) nervous system (Steere, 1989). Neurologic manifestations, known as Lyme neuroborreliosis, occur in 5% to 20% of North American cases and include aseptic meningitis, encephalopathy, facial nerve palsy, radiculitis, and peripheral neuropathy (Pachner et al, 1998a). Carditis occurs in up to 8% of cases (Nagi et al, 1996).

The clinical manifestations of Lyme borreliosis are felt to be the result of the inflammatory response to the presence of spirochetes in tissues (Duray, 1989; Duray and Steere, 1986). The localization of spirochetes in tissues has been documented using nonspecific silver impregnation techniques in the skin of patients with erythema chronicum migrans (Berger, 1984), in the skeletal muscle of patients with myositis (Reimers et al, 1993), and in the synovium of patients with Lyme arthritis (Johnston et al, 1985). All three genospecies of *B. burgdorferi* have been found in the cerebrospinal fluid (CSF) by PCR (Busch et al, 1996). However, there is little data on the localization of *B. burgdorferi* in CNS tissues. This is explained by the paucity of human biopsy and autopsy materials and of animal models of Lyme disease featuring neuroborreliosis. Of the several animal models studied (Barthold, 1991; Pachner et al, 1995a; Pachner and Itano, 1990; Roberts et al, 1995; Schaible et al, 1989), only the nonhuman primate model features consistent CNS infection. Inoc-

Received March 8, 2000.
Supported by a grant from the National Institutes of Health: NIH NS34715.
Dr. Cadavid was supported by the Méndez Fellowship for Clinicians from the American Registry of Pathology and by a grant from the Division of Disadvantaged Assistance, Bureau of Health Professions, Health Resources and Services Administration.
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ulation of nonhuman primates with the N40Br strain of *B. burgdorferi* results in infection of the subarachnoid space (Pachner et al, 1995b; 1995c), CSF pleocytosis (Pachner et al, 1995c), and CNS inflammation as measured by expression of IL-6 and IFN-gamma (Pachner et al, 1997).

The goal of the present study was to investigate the localization and expression of surface proteins of *B. burgdorferi* in tissues. For this, infection was compared in three groups of animals: infected immunosuppressed, infected immunocompetent, and uninfected controls. We found that steroids altered the infection in several ways to the disadavantage of the host. The localization of *B. burgdorferi* in the CNS was the leptomeninges and in the PNS, the endoneurium and connective tissues of nerves and muscles. We also confirmed that there are significant changes in expression of surface proteins during the infection.

Results

Clinical Manifestations

None of the animals in the study (seven infected and two controls) developed any manifestations of disease evident to their veterinary caretakers. Although erythema chronicum migrans has been observed after syringe-inoculation of immunocompetent nonhuman primates in the past (T O'Neill and AR Pachner, unpublished data), this was not seen in the present study. None of the animals inoculated with *B. burgdorferi* showed evidence of CSF leukocytosis.

Specific Serum Antibody Response

All animals inoculated with *B. burgdorferi* showed evidence of anti-*B. burgdorferi* antibodies in serum by ELISA. The data for two of the immunosuppressed and one of the immunocompetent animals is shown in Figure 1. The data for the other two immunosuppressed and two immunocompetent animals shows the same findings (Pachner et al, unpublished data). In immunosuppressed animals, the predominant specific antibody response was of the IgM isotype for the duration of the infection. In immunocompetent animals the initial response was IgM, but it switched to IgG after 3 weeks. No specific antibodies were detected in the two uninfected controls (not shown).

B. Burgdorferi OspA DNA in Tissues

The presence of *B. burgdorferi* in several tissues was studied by ospA PCR-ELISA. The mean and 95% confidence interval (CI) of the mean optical density (OD) for the buffer negative controls was 0.235 (0.22–0.24). The mean (95% CI) OD for the tissue negative controls (animals C1–2) was 0.24 (0.23–0.25). The mean (95% CI) for the 1 pg, 300 fg, 100 fg, 30 fg, and 10 fg positive controls were 0.60 (0.53–0.68), 0.54 (0.45–0.63), 0.43 (0.32–0.47), 0.38 (0.30–0.47), and 0.37 (0.28–0.46), respectively. A comparison of the mean (95% CI) OD in all tissues examined in immunocompetent (A1–3) and immunosuppressed (B1–4) animals is shown in Table 1. The amount of ospA DNA was significantly higher in all tissues of immunosuppressed animals compared with immunocompetent animals. The highest OD in immunosuppressed animals was found in skeletal muscle and the lowest in the brain. The highest OD in immunocompetent animals was also found in skeletal muscle, although it was significantly lower than that in immunosuppressed animals. The highest OD in the CNS was found in the spinal cord of immunosuppressed animals.

Localization of Spirochetes in Tissues

The PCR-ELISA showed that *B. burgdorferi* DNA was present in most tissues examined from all inoculated animals, but the OD indicated the spirochetal load was significantly higher in the immunosuppressed group. To further examine this question, we examined microscopically one paraffin and five frozen sections stained with anti-*B. burgdorferi* polyclonal antibodies looking for spirochetes. About 100 microscopic fields ($\times$200) were examined from each tissue. The results are shown in Table 2. Spirochetes were found only in tissues from immunosuppressed animals, confirming that the spirochetal load in immunocompetent animals was very low.

To determine the localization and approximate number of spirochetes in tissues, we concentrated on the immunosuppressed animals. The number of spirochetes was counted by a masked examiner (DC) in 20 consecutive $\times$200 microscopic fields of paraffin and/or frozen immunostained sections. Tissues from one uninfected animal (C1) were included to control for specificity. The results are shown in Table 3. In the spinal cord, spirochetes were found in the leptomeninges (Fig. 2A), motor and sensory nerve roots (Fig. 2B), and dorsal root ganglia (Fig. 2C), but not in the parenchyma itself. The lumbar roots and cauda equina had the highest number of spirochetes found in the spinal cord. In the PNS, spirochetes were found in the perineurium (Fig. 2D), endoneurium, epimysium, and endomysium. The highest number was found in the endomysium. The number of spirochetes found in skeletal muscle was greater in frozen-unfixed sections than in frozen-fixed or paraffin-fixed sections (Table 3). This demonstrates that antigen retrieval with protease, although effective to unmask spirochetal antigens, underestimates the number of spirochetes in certain tissues. In the heart, spirochetes were found in the connective tissue throughout the pericardium and myocardium. Low numbers of spirochetes were also found in the adventitia and the media of the aorta. The bladder had the highest spirochetal load of all paraffin sections examined. No spirochetes were seen intravascularly, intracellularly, or in clumps in any tissue.

Expression of B. Burgdorferi Proteins in Tissues

To determine if there was a change in spirochetal protein expression during the course of the infection, we studied tissues from immunosuppressed animals by immunohistochemistry with antibodies to flagellin,

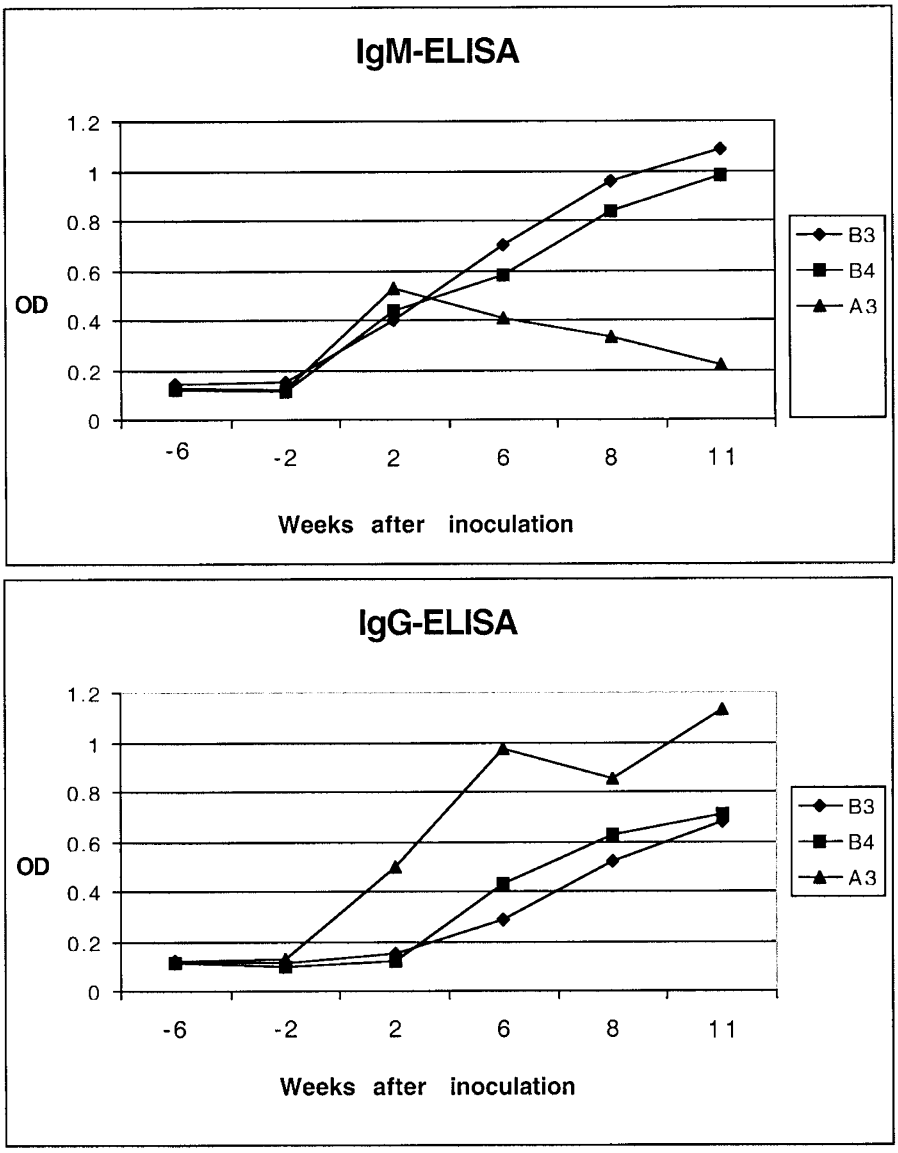

Figure 1.
IgM and IgG-specific antibodies in serum of immunocompetent (A3) and immunosuppressed (B3 and B4) *Macaca mulatta* infected with *Borrelia burgdorferi,* measured by whole-cell sonicate ELISA.

ospA, ospB, and ospC. These four proteins were expressed by the N40Br strain of *B. burgdorferi* at the time of inoculation in culture media (Pachner and Itano, 1990). Sections from uninfected hearts were syringe-inoculated with cultured N40Br spirochetes and used as positive controls. Sections from the hearts of irradiated C3H mice that were killed 7 days after intradermal inoculation of N40Br spirochetes were also used as controls. The results (Table 4) showed expression of flagellin, but not of ospA, ospB, or ospC, in the hearts of all four immunosuppressed animals. Flagellin and ospC, but not ospA or ospB, were expressed in the hearts of irradiated C3H mice 7 days after inoculation. Expression of ospA and ospB was found only in the heart sections syringe-inoculated with cultured spirochetes. Similar results were obtained in skeletal muscle (not shown). These results indicated significant changes in surface protein expression between the time of inoculation and the time of necropsy 4 months later.

Inflammation in Tissues

We compared the presence and severity of inflammation in infected tissues from immunocompetent and immunosuppressed animals using light microscopy of hematoxylin and eosin (H&E)-stained paraffin sections. For this, the severity of inflammation was scored from 0 to 4 by counting the number of inflammatory foci visible at $\times 40$ magnification in 3×2 cm paraffin sections from all available paraffin blocks (Table 5). In most tissues examined from infected animals, the inflammation was absent or minimal. Significant inflammation was found only in the hearts of the four immunosuppressed animals (B1–4) and in one of the immunocompetent animals (A3). In addition to wide-

Table 1. Mean (95% Confidence Interval of the Mean) Optical Densities (OD) of *B. Burgdorferi* ospA by PCR-ELISA in Tissues from Immunocompetent (A1–3) and Immunosuppressed (B1–4) *M. Mulatta* Inoculated Intradermally 4 Months Earlier

Tissue	Immunocompetent	Immunosuppressed	p value[a]
Cerebrum	0.385 (0.33–0.44)[b]	0.49 (0.40–0.57)	0.026
Brainstem and cerebellum	0.365 (0.31–0.42)	0.49 (0.41–0.57)	0.005
Spinal cord	0.370 (0.30–0.44)	0.81 (0.70–0.91)	<0.001
Dura mater	0.302 (0.21–0.40)	0.65 (0.45–0.84)	0.014
Peripheral nerves and plexus	0.371 (0.28–0.46)	0.98 (0.90–1.06)	<0.001
Skeletal muscles	0.431 (0.33–0.53)	1.00 (0.87–1.13)	<0.001
Heart	0.384 (0.31–0.47)	0.97 (0.89–1.05)	<0.001
Bladder	0.362 (0.16–0.56)	0.88 (0.59–1.18)	0.03
Ovary/testis	0.371 (0.28–0.46)	0.96 (0.79–1.12)	0.007

[a] By one tailed *t* student test.
[b] The mean (95% CI of the mean) OD for all tissues examined from uninfected animals (C1–2) was 0.24 (0.23–0.25).

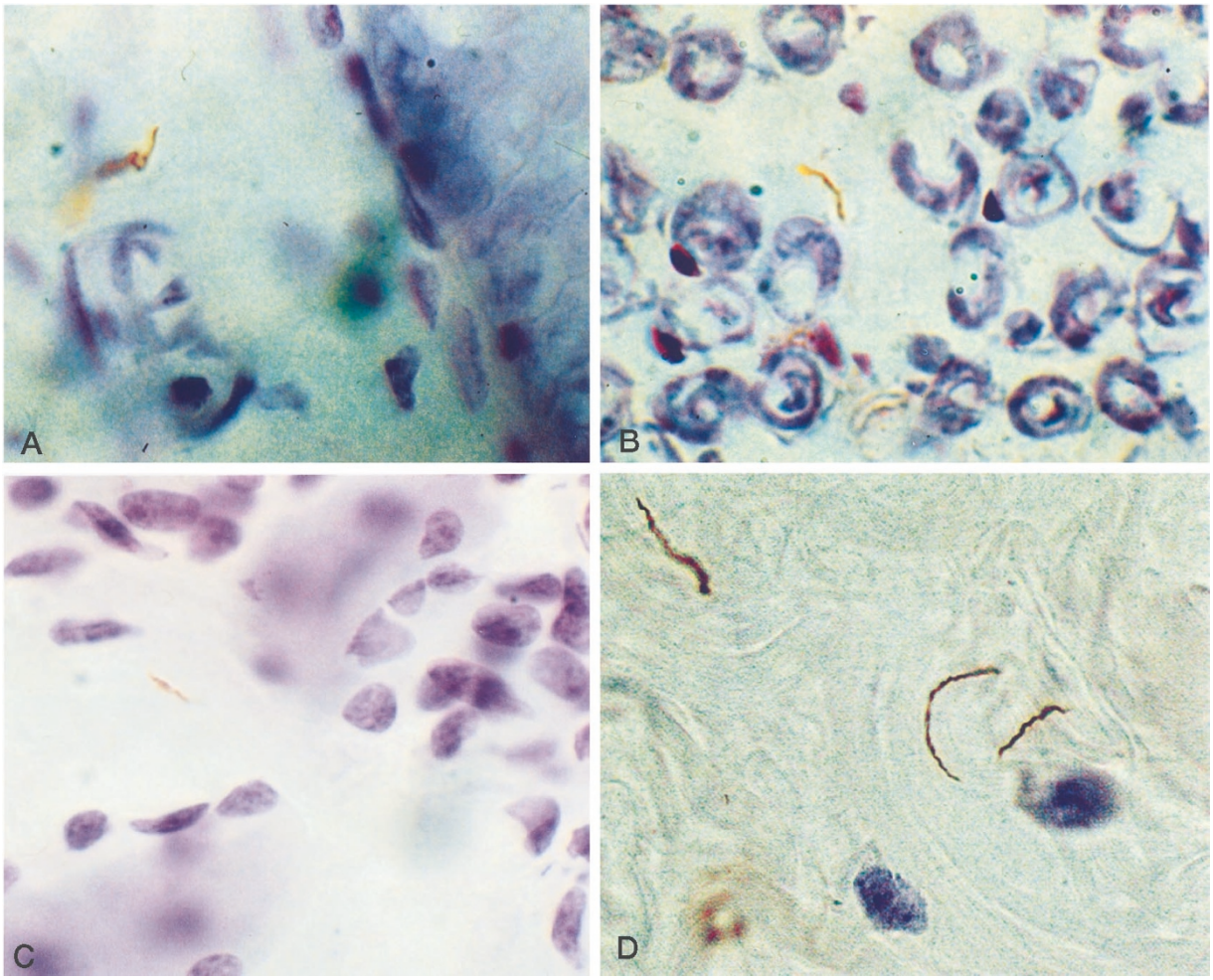

Figure 2.
Localization of *B. burgdorferi* by immunohistochemistry in paraformaldehyde-fixed, frozen tissues from immunosuppressed *M. mulatta*, as follows: A, spinal cord leptomeninges (×900 oil); B, anterior nerve root (×750); C, dorsal root ganglia (×440); D, epineurium (×750).

spread inflammation, the hearts of the immunosuppressed animals had areas of cardiac necrosis and fibrosis and fiber degeneration (Fig. 3, A and B). None of the tissues from the uninfected controls showed any inflammation or evidence of tissue injury (not shown).

Immunohistochemistry with antibodies to CD3 (T cells), CD20 (B cells), P63 (plasma cells), and HAM56 (macrophages) was used to compare the inflammatory infiltrate in the hearts of immunocompetent (A1–3) and immunosuppressed (B1–4) animals (Table 6). One section per tissue per animal was studied. In tissues

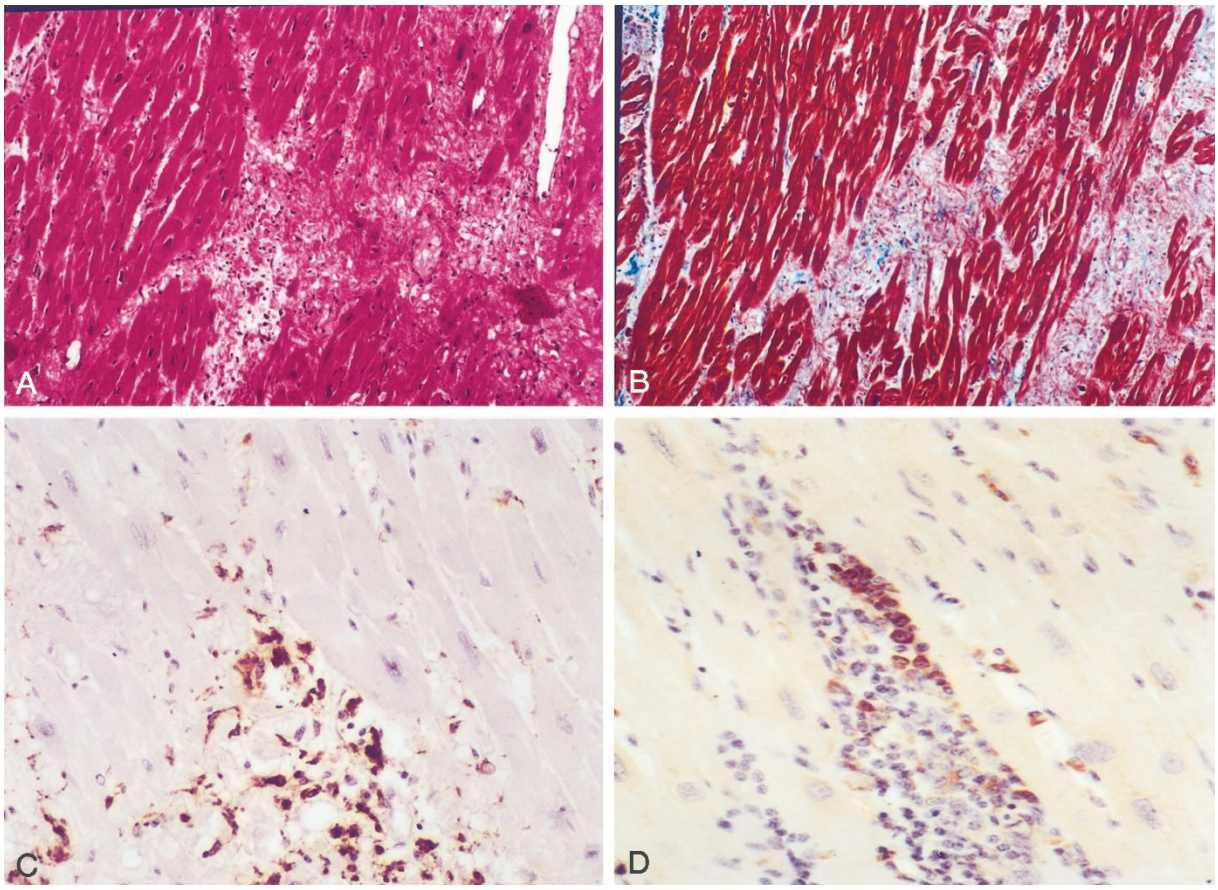

Figure 3.
Heart section from an immunosuppressed animal (B4) stained with hematoxylin and eosin (A, ×100); Gomori trichrome (B, ×100); antimacrophage antibody HAM56 (C, ×400); and antiplasma cell antibody (p63) (D, ×200).

Table 2. Localization of Spirochetes by Immunohistochemistry[a] in Tissues of Immunocompetent (Group A) and Immunosuppressed (Group B) *M. Mulatta* Infected with *B. Burgdorferi* and in Uninfected Controls (Group C)

Group	Brain hemispheres	Spinal cord	Peripheral nerves	Skeletal muscle	Heart	Bladder
A (n = 3)	0/3[b]	0/3	0/3	0/3	0/3	0/3
B (n = 4)	0/3	4/4	4/4	4/4	3/3	2/2
C (n = 2)	0/2	0/2	0/2	0/2	0/2	0/2

[a] One hundred 200× microscopic fields were examined per tissue per animal.
[b] Number of positive animals divided by number of animals examined. Not all tissues were available from all animals.

where more than one paraffin block was available, the one with more inflammation was studied. All sections examined had T cells, B cells, plasma cells, and macrophages. The number of foci was significantly higher for all cell types in the immunosuppressed animals. In the case of the T cells, the sizes of the individual foci were larger in immunocompetent than in immunosuppressed animals (not shown). Large areas of fiber degeneration with abundant macrophages (Fig. 3C) were seen only in immunosuppressed animals. The number of plasma cells (Fig. 3D) was much higher than that of B cells.

Discussion

This study investigated the localization and surface protein expression of *B. burgdorferi* in tissues from nonhuman primates. We used the nonhuman primate model because we were particularly interested in the effects on the nervous system, which cannot be studied in the small animal models. All inoculated animals developed infection, as evidenced by production of specific antibodies in serum and by detection in tissues of ospA DNA by PCR-ELISA and of spirochetes by immunohistochemistry. In addition, *B. burgdorferi* was cultured from the heart of one of the immunosuppressed animals (Pachner et al, 1998b). The data indicates that steroids altered the course of the infection in various ways, including persistence of specific serum antibodies of the IgM isotype, increased spirochetal tissue load, and tissue injury to the heart. The higher spirochetal load of immunosuppressed animals allowed us for the first time to study

Table 3. Number of Spirochetes[a] in Tissues from Infected Immunosuppressed (B1–4) and Uninfected (C1) *M. Mulatta*

Tissues[b]	B1	B2	B3	B4	C1
Cervical cord					
Parenchyma		0.0b			
Roots		0.05 (0.2) b			
Leptomeninges		0.1 (0.3) b			
Thoracic cord					
Parenchyma	0.0a	0.0a	0.0a	0.0a	0.0a
	0.0b	0.0b			0.0b
Roots		0.30 (0.7) a		0.25 (0.5) a	0.0 a
	0.0b	0.20 (0.4) b			
Leptomeninges		0.13 (0.5) a		0.0a	0.0a
	0.0b	0.30 (0.5) b			
Lumbar cord					
Parenchyma	0.0b	0.0b		0.0b	0.0b
Roots	0.0b	1.50 (2.8) b	0.36 (0.5) b	1.0 (1.9) b	0.0b
Leptomeninges	0.0b	0.15 (0.4) b		0.3 (0.5) b	0.0b
Peripheral nerve					
Perineurium				0.0a	0.0a
	3.3 (2.6) b	6.1 (8.9) b			0.0b
Endoneurium		0.3 (1.1) a		0.0a	0.0a
	0.2 (0.6) b	0.0b			
Skeletal muscle	0.05 (0.2) a		0.23 (0.6) a		0.0a
		0.8 (1.1) b	0.80 (1.4) b	0.1 (0.32) b	
			11 (10) c	15 (17) c	
Bladder					
Mucosa			8.3 (7.6) a	7.2 (10.7) a	0.0a
Muscle			32 (36) a	7.2 (8.7) a	0.0a
Heart		1.1 (1.2) a	1.0 (1.7) a	0.6 (0.80) a	0.0a
				1.0 (2.16) b	0.0b
Aorta (adventicia)		0.2 (0.4) b			

[a] Twenty consecutive 200× microscopic fields stained with anti-*B. burgdorferi* polyclonal antibody were counted by a masked examiner; results are given as mean (SD) number of spirochetes/200× field.

[b] The tissues were (a) fixed in formalin and embedded in paraffin, (b) fixed in paraformaldehyde and frozen, and (c) frozen unfixed.

Table 4. Expression of *B. Burgdorferi* Proteins by Immunohistochemistry in Hearts of Immunosuppressed *M. Mulatta* 4 Months after Inoculation, in Irradiated C3H Mice 7 Days after Inoculation, and in Cultured Spirochetes[a]

Protein	M. mulatta	C3H mice	Cultured spirochetes
Flagellin	+	+	+
OspA	−	−	+
OspB	−	−	+
OspC	−	+	+

[a] Hearts from uninfected C3H mice were syringe-inoculated with cultured spirochetes and fixed in methanol.

the localization of *B. burgdorferi* in the nervous system and to compare the number of organisms in different regions of the same tissue.

The clinical manifestations of Lyme borreliosis are believed to be the result of the inflammatory response to infection by *B. burgdorferi* (Barbour and Fish, 1993; Steere, 1993). Persistence of inflammation with or without residual infection may explain why some patients with Lyme borreliosis remain symptomatic despite treatment with adequate doses of antibiotics (Carlson et al, 1999; Hansen and Lebech, 1992; Oksi et al, 1999; Steere et al, 1983). In addition to antibiotics, Lyme borreliosis patients are sometimes given steroids to treat pain syndromes (Oschmann and Dorndorf, 1995), hasten the resolution of facial palsy (Hyden et al, 1993; Sigal, 1992), or treat late neurologic complications of chronic infection (Kruger et al, 1990). Steroids may also be given to patients with Lyme borreliosis to treat other inflammatory diseases (Federlin and Becker, 1989) or patients with idiopathic inflammatory syndromes undiagnosed as Lyme borreliosis (Steere et al, 1977). Little is known about the effects of steroids on the course of infection with *B. burgdorferi*. One study in mice showed that administration of steroids can decrease the arthritis (Hurtenbach et al, 1996). Another study in dogs showed that treatment with steroids can reactivate latent infection (Straubinger et al, 1998). It was likely that steroids, by impairing the host's ability to clear the infection, would increase the spirochetal tissue load, as has been demonstrated in other infections (Guimaraes et al, 1997).

The finding that treatment with steroids resulted in persistence of high levels of specific antibodies of the IgM isotype was unexpected. Previous studies had shown that although treatment with dexamethasone

Table 5. Severity of Inflammation[a] in Tissues of Immunocompetent (Group A) and Immunosuppressed (B) *M. Mulatta* Infected with *B. Burgdorferi* and in Uninfected Controls (C)

Monkey[c]	Brain	Spinal cord	Peripheral nerve	Skeletal muscle	Heart	Bladder
A1	0, 0, 0[b]	0, 0, 0	0	1, 0	1, 0, 0, 0,	1
A2	0, 0, 0	0, 0, 0	1	1, 0	1, 1	1
A3	0, 0, 0	0, 0,	1	0, 0	3, 1, 3	na[c]
B1	0, 0, 0	0, 0, 0	0	0, 0	3	na
B2	0, 0, 0	0, 0, 0	1, 0	0, 0	3, 3, 2, 1	na
B3	0, 0, 0	0, 0,	1	1, 0	3, 2	1
B4	0, 0, 0	0, 0, 0	0	1, 0	3, 3, 1	2
C1	0, 0, 0	0, 0, 0	0	0, 0	0, 0, 0	0
C2	0, 0, 0	0, 0, 0	0	0, 0	0, 0	0

[a] 3 × 2 cm H&E-stained paraffin sections were examined microscopically at 40× magnification and scored based on the number of inflammatory foci as: 0 = no foci; 1 = 1–3 foci; 2 = 4–10 foci; 3 = 11–20 foci; 4 = > 20 foci.

[b] More than one paraffin block was available for examination from some tissues.

[c] na = not available.

Table 6. Characterization of the Inflammatory Foci[a] in the Hearts of Immunocompetent (A1–3) and Immunosuppressed (B1–4) *M. Mulatta* Infected with *B. Burgdorferi*

Animal	T cells (CD3)	B cells (CD20)	Plasma cells (P63)	Macrophages (HAM56)
A1	1[b]	1	1	1
A2	1	1	1	1
A3	2	1	3	2
B1	4	1	3	4
B2	4	1	4	4
B3	2	2	4	4
B4	3	4	4	4

[a] 3 × 2 cm paraffin sections, immunostained for each of the markers, were examined microscopically at 40× magnification and scored based on the number of foci as: 0 = no foci, 1 = 1–3 foci, 2 = 4–10 foci, 3 = 11–20 foci, 4 = > 20 foci.

[b] Only the paraffin block with the highest score on H&E examination was examined from each animal.

greatly reduces the number of B cells in the spleen, it does not affect their capacity to switch from IgM to IgG in vitro (Sabbele et al, 1987; Saether et al, 1998). Our data indicate that high levels of specific serum antibodies of the IgM isotype were unable to eradicate the infection from any of the tissues examined. This is consistent with a previous study using another spirochetal pathogen, *Borrelia hermsii*, in which specific antibodies of the IgM isotype eliminated spirochetes from the blood, but not from the CNS, of infected irradiated mice (Cadavid et al, 1993).

Until now the localization of *B. burgdorferi* in CNS tissues was unknown. Studies in humans and experimental animals suggested that the most likely localization was leptomeningeal (Nocton et al, 1996; Pachner et al, 1995c). Our studies confirmed the observation that expression of ospA is down-regulated early after infection (Fikrig et al, 1998; Zhong et al, 1997) and provide, for the first time, evidence that expression of ospC is down-regulated in chronic infection, while expression of flagellin is maintained. Our results also support the view that spirochetes are present in low numbers in tissues from an immunocompetent host. Another group examined tissues of immunocompetent nonhuman primates, 6 months after tick-inoculation with *B. burgdorferi,* and failed to find any spirochetes (Roberts et al, 1995). Our PCR-ELISA was able to detect as little as 10 to 30 fg of *B. burgdorferi* DNA per 500 ng of tissue DNA. Based on the assumptions that the size of the *B. burgdorferi* genome is approximately 1.4 million base pairs (bp) (Fraser et al, 1997), that there are approximately 16 genomes per borrelia cell (Hinnebusch and Barbour, 1992; Kitten and Barbour, 1992), that the weight of 9×10^5 bp of DNA is approximately 1 fg (Sambrook et al, 1989), one can estimate there are approximately 25 fg of DNA per borrelia cell. Based on the assumption that the yield of DNA extraction from mammalian tissues is approximately 1 mg DNA per mg of tissue (Sambrook et al, 1989), one can estimate that the sensitivity of the PCR-ELISA was approximately 2 cells per μg of tissue (10–30 fg of *B. burgdorferi* DNA per 500 ng of tissue DNA). The mean OD for most tissues from immunocompetent animals was closer to the mean OD for the 10 to 30 fg positive controls, suggesting that there were approximately one to four cells per μg of tissue. In contrast, the tissue from immunosuppressed animals with the lowest mean OD was the brain (0.49), which is between the mean OD of the 100 and 300 fg positive controls, or 8 to 24 cells per μg of tissue. The sensitivity of immunohistochemistry was considerably lower than that of the PCR-ELISA. It was able to detect spirochetes only in tissues with high OD (> 0.80) and failed to find spirochetes in the brain (OD 0.49) and dura mater (OD 0.65), which were positive by PCR-ELISA. It is likely that a more intense search

for spirochetes, preferably using unfixed sections from PCR-positive tissues, will eventually reveal the location of spirochetes in immunocompetent hosts. Using electron microscopy, spirochetes have been already found in the hearts of immunocompetent mice (Pachner et al, 1995a).

Our finding of spirochetes in the leptomeninges and nerve roots, but not in the CNS parenchyma, is consistent with the clinical presentation of Lyme neuroborreliosis. The most common manifestations of Lyme neuroborreliosis are headache, meningismus, or radiculopathic symptoms, whereas CNS parenchymal involvement is rare (Hansen and Lebech, 1992; Pacher et al, 1998a). The localization of spirochetes in the CNS of humans has been reported only in a handful of cases using nonspecific silver impregnation techniques (Kobayashi et al, 1997; Kuntzer et al, 1991; Miklossy et al, 1990). The positivity of the PCR-ELISA in samples from the cerebrum and brainstem likely represents leptomeningeal, rather than parenchymal, localization. The localization of spirochetes in the CNS has been studied in other spirochetal diseases. In tick-borne relapsing fever, an acute or sub-acute disease that frequently involves the CNS (Cadavid, 1998a), the spirochetes are found mainly in the brain parenchyma perivascularly and in the meninges. In paretic neurosyphilis, treponemes are identified in the cortex of 25% to 40% of brains examined at autopsy, mainly in the gray matter of the frontal areas, and are difficult to find after treatment (Cadavid, 1998b). Treponemes in the brain can be found in three patterns: diffusely scattered, in dense focal conglomerates, and perivascularly in masses or as single specimens. The diffusely scattered pattern appears to be the most frequent. Treponemes are also occasionally found in meningeal neurosyphilis, free floating in CSF, and rarely in gummatous and tabetic neurosyphilis (Cadavid, 1998b).

Our finding of the localization of *B. burgdorferi* in non-CNS tissues is consistent with prior observations in humans and small animal models. Spirochetes were observed by silver staining in muscle biopsies of patients with Lyme myositis (Hoffmann et al, 1995; Reimers et al, 1993) and in perineural and muscle connective tissue of mice (Barthold, 1991). To our knowledge this is the first report of the finding of *B. burgdorferi* in the endoneurium. Spirochetes have also been observed in cardiac muscle from humans (de Koning et al, 1989; Duray, 1989; Klein et al, 1991) and experimental animals (Armstrong et al, 1992; Campbell et al, 1994; Pachner et al, 1995a), and in bladders of experimental animals (Campbell et al, 1994; Lebech et al, 1995).

It is unclear why, of all the infected organs, only the heart had significant inflammation. The inflammatory infiltrate in the heart had multiple foci of T cells and plasma cells and, to a lesser degree, B cells. The results suggest that an increase in the immune response secondary to an increase in the spirochetal load in steroid-treated animals caused injury to the heart. The large numbers of macrophages in the hearts of immunosuppressed animals confirmed the presence of cardiac fiber degeneration. The fact that tissue injury occurred in the heart, but not in other infected tissues, suggests that injury was caused by inflammation. There have been few studies of inflammation in tissues of humans with Lyme borreliosis. In one patient, a lymphocytic and plasma cell infiltrate of the meninges was found (Duray and Steere, 1986). In one advanced case, the leptomeningeal infiltrate extended to the white and gray matter (Meures et al, 1990). Examination of nerve roots and their ganglia in another case revealed mononuclear inflammation (Duray and Steere, 1986). Muscle biopsies had shown focal interstitial inflammation with lymphocytes and plasma cells in the vicinity of small vessels, and occasional mild myofiber degeneration (Reimers et al, 1993). Nerve biopsies had shown vasculitis of epi, peri, and endoneurial vasa nervorum with lymphocytes and plasma cells, and occasional angiopathic lesions in the neural parenchyma (Camponovo and Meier, 1986; Duray, 1987; Meier and Grehl, 1988). Inflammation in the heart has also been observed in humans (Duray and Steere, 1986) and experimental animals (Schaible et al, 1990). One group working with immunosuppressed mice observed cardiomyopathy with mononuclear inflammatory infiltration and fibrillary necrosis (Defosse et al, 1992). Although we did not have a control group of uninfected monkeys treated with steroids, it is unlikely that injury to the heart was caused by steroids. In a study of experimentally infected ponies treated with steroids, none of the tissues examined in uninfected controls had any histologic alterations (Chang et al, 2000).

The large number of organisms in tissues from immunosuppressed animals gave us for the first time the opportunity to study the localization of *B. burgdorferi* in the CNS compared with other tissues, and to confirm that significant changes in expression of surface proteins occur during the infection. The data presented above also suggest that administration of steroids without antibiotics can cause injury in tissues with inflammation. The mechanism of this tissue injury remains to be determined.

Materials and Methods

Animals and Tissues

Adult *Macaca mulatta* were inoculated intradermally with 1 million spirochetes of the N40Br strain of *B. burgdorferi,* as previously described (Pachner et al, 1995c). *B. burgdorferi* was grown in modified Kelly's medium. Three groups of monkeys were used for these experiments: Groups A ($n = 3$) and B ($n = 4$) were inoculated with *B. burgdorferi*, and Group C ($n = 2$) consisted of uninfected control animals. Group B was immunosuppressed with 2 mg/kg/day of oral dexamethasone for 1 week before inoculation, followed thereafter by 1 mg/kg/day until necropsy. The housing and care was in accordance with the *Animal Welfare Act and the Guide for the Care and Use of Laboratory Animals* (Department of Health and Human Services Publication No. 85–23, 1985) in facilities

accredited by the American Association for Accreditation of Laboratory Animal Care. Before initiation, the study was reviewed and approved by the Georgetown University Animal Care and Use Committee. Blood and CSF were collected periodically to test for the presence of anti-*B. burgdorferi* antibodies in serum and of CSF pleocytosis. Euthanasia and necropsy were performed 4 months after inoculation as described (Pachner et al, 1995b), with the addition of cardiac exanguination and total body perfusion with buffer to minimize blood contamination of tissues. The following tissues were removed at autopsy: frontal, parietal, temporal, and occipital brain cortex; cervical, thoracic, and lumbar cord; brachial plexus and median and sciatic nerves; biceps and quadriceps skeletal muscles; left ventricular myocardium; and bladder. Tissues were processed by one or more of the following methods: (a) routine formalin-fixation and paraffin-embedding; (b) fixation in 4% paraformaldehyde for 6 hours at 4° C, dehydration in 25% sucrose in PBS pH 7.4 at 4° C for 3 days followed by snap-freezing with Ultrafreeze spray (Fisher Scientific, Houston, Texas), and embedding in OCT (Tissue-Tek, Torrance, California) compound; and (c) snap-freezing in dry-ice-cold ethanol, embedding in OCT compound (Tissue-Tek), and fixation of sections in 100% ethanol for 5 minutes. Frozen tissues were stored at −80° C. Paraffin-sections were sectioned at 5 microns and frozen sections at 8 microns.

ELISA

ELISA was performed as described (Pachner et al, 1998b). In brief, the antigen was sonicated of the strain N40Br. Two hundred microliters of antigen coating solution were added to a microtitration plate (Linbro Scientific, Hamden, Connecticut) at a concentration of 5 micrograms/ml and incubated overnight at 4° C. Plates were washed three times with PBS 0.05% Tween 20, and 200 μl of the sera were added, at 1/500 dilution. Plates were incubated for 2 hours at 37° C, then washed again as described above. Two hundred microliters of horseradish peroxidase-conjugated goat antihuman immunoglobulin, isotypes G and M (Organon Teknika-Cappel, Malvern, Pennsylvania) were diluted 1:10,000 in PBS-Tween 20, and added to each well. Incubation followed for 2 hours at 37° C. Plates were washed, and 200 microliters of TMB Microwell Peroxidase Substrate (Kirkegaard and Perry Labs, Gaithersburg, Maryland) were added to each well, immediately after which 50 microliters of 8% sulfuric acid were added to stop the reaction. The plates were read immediately on an ELISA spectrophotometer (BioRad, Richmond, California) at 450 nm. On each plate, a standard positive control was run within its linear range of dilutions. All serum samples were performed in duplicate.

PCR-ELISA

Analysis of infection in tissues was performed by PCR-ELISA as previously described. Minced tissues were treated with proteinase K, extracted with phenol-chloroform and precipitated with ethanol. OD 260/280 ratios of the extracted DNA prior to PCR were required to be 1.5 or greater. All reagents were from Boehringer-Mannheim (Indianapolis, Indiana) unless otherwise stated. Five hundred nanograms of tissue DNA were used as template; digoxygenin-11-UTP, 0.01M, was one of the nucleotides added to the PCR reaction mixture. PCR products were subjected to hybridization with the appropriate biotinylated probe (biotinylation performed at Lofstrand Laboratories, Gaithersburg, Maryland) and subsequently captured onto a streptavidin-coated plate. The PCR product bound on the plate was detected by addition of an anti-digoxygenin antibody conjugated to alkaline-phosphatase with subsequent color development with substrate. The following probes and primers for the ospA *B.burgdorferi* gene were used in the PCR-ELISA:

ospA149: 5′-TTATGAAAAAATATTTATTGGGAAT
ospA319: 5′-CTTTAAGCTCA AGCTTGTCTACTGT
Probe- ospAwt3: 5′-AGCGTTTCAGTAGATTTGCCT-GCTGGTG

Tubes without DNA were used as buffer negative controls. Tissues from uninfected animals served as a negative control for tissue DNA, and nonhuman primate DNA containing serial dilutions of *B. burgdorferi* DNA (1 pg, 300 fg, 100 fg, 30 fg, and 10 fg per 500 nanograms of tissue DNA) served as positive controls for infected tissues. All assays were performed in duplicate.

Light Microscopy

All tissue sections were examined with standard light microscopy by an investigator (DC) masked to the infectious and immune status. The spirochetal load was measured counting the total number of spirochetes in each of twenty ×200 consecutive fields in paraffin and/or frozen sections after immunostaining (see below). Spirochetes were defined by their characteristic spiral morphology and positive staining with the DAB chromogen. Inflammation was defined by the characteristic morphology of mononuclear inflammatory cells on H&E staining, and by positive DAB staining after immunohistochemistry for specific cell markers. To compare the severity of inflammation in different tissues, one 3 × 2 cm section from each paraffin block stained with H&E was examined at low magnification (×40) and scored as follows: 0 = absent; 1 = minimal (1–3 foci); 2 = mild (4–10 foci); 3 = moderate (11–20 foci); and 4 = severe (> 20 foci). In some tissues (brain, spinal cord, and heart) more than one paraffin block was available for examination. The same scoring method was used with sections immunostained for specific inflammatory markers (see below). Sections from the heart were also stained with Gomori's trichrome.

Immunohistochemistry

Precleaned superfrost glass slides (Fisher Scientific) were used for immunohistochemistry. A three-step

streptavidin-peroxidase technique was used to determine the localization of proteins in tissue sections. All reactions were performed at room temperature manually or in an automatic immunostainer (Biogenex, San Ramon, California). Nonspecific binding was reduced by blocking slides with Biogenex blocking solution for 15 minutes. Ten percent pre-inoculation monkey serum was used to block non-specific binding in the leptomeninges and dura mater. Endogenous peroxidase activity was reduced by incubation with 3% H_2O_2 for 20 minutes at room temperature. Formalin-fixed sections were treated with 0.5 mg/ml protease type VIII (Sigma P-5380; Sigma Chemical, St. Louis, Missouri) for 10 minutes for antigen retrieval. A 1:1 $\times$ 10^3 dilution of hyperimmune rabbit serum (Pachner et al, 1994) was used for detection of *B. burgdorferi.* This hyperimmune serum has high titers of anti-*B. burgdorferi* antibodies by ELISA and Western blot (not shown). For the localization of flagellin, ospA, ospB, and ospC, we used mouse monoclonal antibody H9724 (Barbour et al,1986a), anti-ospA (H5332) and anti-ospB mouse (H614) monoclonal antibodies (Barbour et al, 1986b), and hyperimmune rabbit serum, specific for recombinant ospC (Fingerle et al, 1995). Commercially available monoclonal antibodies (Dako, Carpinteria, California) were used for identification of T cells (Dako's A0452 aCD3 in 1:1 $\times$ 10^3 dilution), B cells (Dako's M0755 aCD20 in 1/200 dilution), plasma cells (Dako's M7077 ap63 in 1/200 dilution), and macrophages (Dako's M0632 aHAM56 in 1/200 dilution). The secondary reagent was a biotinylated goat antirabbit or antimouse polyclonal antibody (Biogenex). The tertiary reagent was horseradish peroxidase-labeled streptavidin (Biogenex). Incubation times were 30 minutes for the primary antibody and 20 minutes for the secondary and tertiary reagents. The chromogen was 3,3 diaminobenzidine tetrahydrochloride (DAB) in 0.24% H_2O_2 for 5 to 15 minutes. The counterstain was Mayer's Hematoxylin for 1 minute. Each incubation was separated by three washes with OptiMax Wash buffer (Biogenex). Tissue sections from uninfected animals were used as negative controls. Sections from tissues inoculated with cultured spirochetes were used as positive controls for localization of whole spirochetes and ospA, ospB, ospC, and flagellin. Sections from monkey spleen were used as positive controls for localization of T cells, B cells, plasma cells, and macrophages. Isotype-matched irrelevant antibodies were used as negative controls in all experiments.

Statistical Analysis

Results for the PCR-ELISA were expressed as mean and two-sided 95% CI of the mean OD. Differences in mean OD between immunosuppressed and immunocompetent animals were compared with one-tailed *t* test. One-tail was used because the spirochetal load was expected to be higher (but not lower) in immunosuppressed animals.

Acknowledgements

We thank Alan G. Barbour (University of California, Irvine, California) and D. Denee Thomas (The University of Texas Health Science Center at San Antonio, Texas) for providing monoclonal antibodies H9724, H614, and H5332; Bettina Wilske (Max von Pettenkofer Institut fur Hygiene und Medizinische Mikrobiologie, Lehrstuhl Bakteriologie, LMU Munich, Germany) for providing rabbit polyclonal antibody to ospC; Melanie Barlett (UMDNJ-New Jersey Medical School, New Jersey) for technical assistance; and Hernando Mena (Armed Forces Institute of Pathology, Washington, DC) for his support.

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