Status of *Borrelia burgdorferi* Infection after Antibiotic Treatment and the Effects of Corticosteroids: An Experimental Study

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Sixteen specific-pathogen-free beagles were infected with *Borrelia burgdorferi*. Three groups of 4 dogs were treated with antibiotics for 30 consecutive days starting 120 days after tick exposure; 4 dogs were untreated controls. At day 420 after tick exposure and again before euthanasia, 2 dogs of each group were treated with prednisone for 14 days. All dogs contracted infection and 11 developed acute arthritis 50–120 days after exposure. After day 120, one of 12 antibiotic-treated dogs and 2 of 4 untreated dogs became lame. Antibiotic therapy reduced the frequency of *Borrelia*-positivity in subsequent skin biopsy samples. After prednisone treatment, both control dogs developed severe polyarthritis. At euthanasia, single tissues of the antibiotic-treated dogs and multiple tissues of all control dogs were *Borrelia*-positive by polymerase chain reaction. Viable spirochetes were not recovered from antibiotic-treated dogs. Two antibiotic-treated dogs showed histologic evidence of minimal lesions, whereas all control dogs had mild polyarthritis with periarteritis.

Lyme disease is caused by the tick-transmitted bacterium *Borrelia burgdorferi,* a spirochete that infects a wide range of mammals and birds, including humans, dogs, cats, cows, horses, and blackbirds [1–4]. After transmission of the bacterium from the tick into the mammalian host, infection is established in the skin, and from there the spirochete disseminates through the body [5]. During the course of the infection, a variety of organs can be affected, especially the skin, large joints, the heart, and the central and peripheral nervous system. Early acute symptoms may be followed by chronic disorders [6].

Many studies have shown that one reason for the perpetuation of the disease is the persisting infection with *B. burgdorferi* despite a strong antibody response by the host [7–9]. Once infection has been established, antibodies against *B. burgdorferi,* induced either by infection or by immunization, are not sufficient to eliminate the organism from the host's tissue [10]. Antibiotic therapy should offer an alternative method to eradicate the bacterium from tissues. Penicillins and tetracyclines are widely used in human and veterinary medicine to treat patients with Lyme disease. However, conflicting reports, mostly based on retrospective studies of natural disease [11, 12] or on short experimental trials in rodents [13], have provoked intense debate as to whether antibiotic therapy can eliminate the organisms and so prevent subsequent relapses.

In this study, we report on the effect of orally (azithromycin, doxycycline) or intravenously (ceftriaxone) administered antibiotics on a well-established and monitored infection with *B. burgdorferi* in beagle dogs. All 3 antibiotics have been used in the treatment of Lyme disease. Azithromycin, a macrolide, and doxycycline, a tetracycline, were chosen because these drugs diffuse into the extracellular and intracellular spaces of the host tissue. Ceftriaxone, a third-generation cephalosporin that is given intravenously but tends to accumulate in the extracellular space of the tissue, was evaluated because it is commonly used when neurologic symptoms are apparent and high therapeutic plasma levels are desired in an attempt to allow the drug to cross the blood-brain barrier [14]. In addition, we studied the effects of the corticosteroid prednisone to see whether therapeutically induced immunosuppression reactivates Lyme arthritis in infected untreated dogs as well as in antibiotic-treated dogs.

We have chosen to work with the dog model because it closely resembles human Lyme borreliosis in many aspects. As we have reported in earlier publications [5, 15], dogs infected with *B. burgdorferi* develop clinically apparent acute arthritis and harbor the organisms indefinitely despite mounting a vigorous antibody response. This long-term controlled study using the canine model gave us access to a large number of sequentially collected tissue and blood samples, the basis for a comprehensive study.

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Materials and Methods

Dogs and tick exposure. Specific-pathogen-free beagles of both sexes from the James A. Baker breeding colony, Cornell University, were used for the study. After being weaned, 16 puppies were transferred into P2-units. At 6 weeks of age, dogs were exposed to *B. burgdorferi*-bearing ticks, which were collected in Westchester County, New York, a week before the first exposure. On day 0 of the experiment, 15 female and 7 male ticks (*Ixodes scapularis*) were placed on the left chest of each dog as described elsewhere [15]. Ticks were allowed to engorge completely and were removed on day 7. At the same time, dogs were immunized against canine distemper virus (CDV strain Rockborn, Dr. Appel, Cornell University) and canine parvovirus (CPV, Dr. Carmichael, Cornell University). To ensure that all dogs became infected, we repeated the exposure with the same number of ticks starting on day 14. Throughout the experiment, infection with *B. burgdorferi* was monitored serologically with serum samples taken at 2-week intervals and by culture and polymerase chain reaction (PCR) by use of skin-punch biopsy samples taken near the site of tick infection at 4-week intervals. Dogs were fed with commercial dog food and water ad libitum. Dogs were maintained in study groups of 4. Clinical signs and body temperature were recorded daily, and body weight, weekly. All experiments were conducted in compliance with regulations of the Animal Welfare Act and of the New York State Department of Health.

Treatment with antibiotics and testing for antibiotic levels in plasma. Starting on day 120 of the experiment, 12 dogs were treated with antibiotics for 30 consecutive days (table 1). Four dogs (A96-4/3, A96-4/5, A96-4/7, A96-4/10) were treated with 25 mg/kg azithromycin (Zithromax; Pfizer Labs, New York, NY) orally once a day; 4 dogs (A96-4/8, A96-4/9, A96-4/11, A96-4/12) were treated with 25 mg/kg ceftriaxone (Rocephin; Hoffmann-La Roche, Nutley, NJ) intravenously once a day; 4 dogs (A96-4/1, A96-4/2, A96-4/4, A96-4/6) received 10 mg/kg doxycycline (doxycycline hyclate capsules; Danbury Pharmacal Inc., Danbury, CT) orally twice a day; and 4 dogs (A96-5/1, A96-5/2, A96-5/3, A96-5/4) received no treatment and were used as controls. During the course of antibiotic therapy, blood samples of all treated dogs were drawn into ethylenediaminetetraacetic acid (EDTA)–coated collection tubes 5 min, 45 min, 1.5 h, 3 h, 6 h, and 12 h after the administration of the drugs on the third, fifteenth, and thirtieth day of treatment. An additional 24-h sample was taken from azithromycin- and ceftriaxone-treated dogs. Plasma was collected after centrifugation (250 *g*, 30 min). Plasma levels of all antibiotics were determined by an agar diffusion bioassay as described by Bennet et al. [16]. *Micrococcus luteus* (ATCC, Manassas, VA) in antibiotic medium 11 (Difco, Becton Dickinson, Franklin Lakes, NJ) was used to test for azithromycin; *Bacillus stearothermophilus* (Difco) in antibiotic medium 4 (Difco) was used to test for ceftriaxone; and *Bacillus cereus* (Difco) in Mueller Hinton medium (Difco) was used to test for doxycycline. Growth inhibition values of the test samples were compared with those of the standard curves created with azithromycin (gift from Pfizer Labs), ceftriaxone (Hoffmann-La Roche), and doxycycline hydroxychloride (Sigma) diluted in plasma of untreated, uninfected dogs. Plasma concentrations of antibiotics were recorded as micrograms per milliliter.

Treatment with prednisone and testing for cortisol/prednisolone levels in plasma. Starting on day 420 after the first tick exposure (271 days after the 30-day antibiotic treatment had ended), 6 dogs previously treated with antibiotics (2 of each treatment group) received oral prednisone for 14 consecutive days. The same treatment was applied to 2 untreated control dogs 413 days after their first tick exposure (table 1). Each dog was treated with 2 mg prednisone per kg body weight twice a day at 9 am and 9 pm. During the course of the corticosteroid treatment (seventh and fourteenth day of treatment), blood samples of all prednisone-treated and control

Table 1. Tick exposure, antibiotic therapy, prednisone treatment, and euthanasia for 12 dogs infected with *Borrelia burgdorfia*.

			Treatment			
Dog no.	Sex	TE, days 0–7, 14–21	Antibiotic, days 120–149	Corticosteroid, days 420–433 or 413–426[a]	Corticosteroid, 14 days before necroscopy	Day of necropsy (after TE)
A96-4/3	F	+	Azithromycin	None	None	513
A96-4/5	F	+	Azithromycin	Prednisone	None	527
A96-4/7	F	+	Azithromycin	Prednisone	Prednisone	588
A96-4/10	F	+	Azithromycin	None	Prednisone	589
A96-4/8	M	+	Ceftriaxone	None	None	517
A96-4/9	F	+	Ceftriaxone	Prednisone	None	531
A96-4/11	M	+	Ceftriaxone	Prednisone	Prednisone	600
A96-4/12	F	+	Ceftriaxone	None	Prednisone	601
A96-4/1	M	+	Doxycycline	None	None	520
A96-4/2	M	+	Doxycycline	Prednisone	None	534
A96-4/4	M	+	Doxycycline	Prednisone	Prednisone	603
A96-4/6	M	+	Doxycycline	None	Prednisone	604
A96-5/1	F	+	None	None	None	505
A96-5/2	M	+	None	Prednisone	None	518
A96-5/3	M	+	None	Prednisone	Prednisone	580
A96-5/4	M	+	None	None	Prednisone	581

NOTE. Dosages: azithromycin: 25 mg/kg orally once a day; 30 days; ceftriaxone: 25 mg/kg intravenously once a day; 30 days; doxycycline: 10 mg/kg orally twice a day; 30 days; prednisone: 2 mg/kg orally twice a day; 14 days. F, female; M, male; TE, tick exposure; +, positive.

[a] Dogs treated with antibiotics, days 420–433; control dogs, days 413–426.

dogs were drawn into EDTA-coated collection tubes 45 min after dosing. The combined levels of cortisol, the naturally occurring active corticosteroid, and prednisolone, the biologically active metabolite of prednisone, were measured in plasma samples with an ELISA and compared with cortisol plasma samples of the same dogs taken before treatment (Diagnostic Laboratory, Cornell University). To determine whether corticosteroids had an effect on the number and disposition of *B. burgdorferi* organisms in the host, we dosed a total of 8 dogs (2 antibiotic-treated dogs of each treatment group and 2 untreated control dogs) with oral prednisone as outlined earlier, starting 14 days before the euthanasia (table 1).

Serology. Serum samples were collected from all dogs at 2-week intervals beginning at day 0 of the experiment. Sera were tested for *B. burgdorferi* antibody levels by a computerized kinetic enzyme-linked immunosorbent assay (KELA), and specific antibodies were detected by Western blotting as described elsewhere [17]. To avoid fluctuation between tests, we tested all sera from each dog at the same time.

Synovial fluids and cerebrospinal fluid (CSF). During the necropsy (table 1), synovial fluids from shoulders, elbows, carpi, and knees were aspirated into a 3-mL syringe with a 20-gauge needle (0.2 mm × 25 mm; Becton Dickinson, NJ). Volumes were recorded and smears for differential cell counts were prepared. Subsequently, synovial fluids were diluted 1 : 10 in Hank's balanced salt solution, and used for total cell counts in a hemocytometer. CSF was obtained with a 20-gauge spinal needle (0.9 mm×38 mm; Becton Dickinson) by percutaneous puncture of the meninges through the spatium atlanto-occipitale. After removal of the stylet, CSF was slowly aspirated into a 3-mL syringe. Total cell counts were obtained with a hemocytometer and differential cell counts from cytospins.

Histopathologic analysis. Dogs were euthanized between day 505 and 604 of the experiment (up to 455 days after the conclusion of antibiotic therapy). Tissues were removed from euthanized dogs, fixed in 10% buffered formalin, trimmed, embedded, cut, and stained with hematoxylin and eosin by standard procedures. Tissues examined for histologic lesions included 6 lymph nodes (superficial cervical, axillary, and popliteal lymph nodes from each side), 10 joints (shoulders, elbows, carpi, knees, and tarsi), meninges, cerebrum, cerebellum, and pericardium from each dog. Tissues were examined by the pathologist without knowledge of the protocol for each animal.

Isolation of B. burgdorferi *from tissue samples.* For isolation of *B. burgdorferi,* skin-punch biopsies (4-mm diameter) were collected by sterile methods while dogs were under local anesthesia at 4-week intervals before and after antibiotic therapy. In addition, 25 different tissues were collected from each dog at necropsy; instruments were changed frequently to avoid cross-contamination. Tissues included skin from left and right sides, synovial membranes from 6 joints (shoulder, elbow, and knee), muscle and fascia from front (musculus triceps and fascia antebrachii) and hind limbs (musculus adductor and fascia lata), superficial cervical, axillary, and popliteal lymph nodes, pericardium, peritoneum, and meninges. Skin biopsy samples were ground in 0.2 mL of BSK-II + KR (with kanamycin and rifampicin) medium with a pellet pestle and placed into 6.5 mL of medium, as reported elsewhere [15]. Postmortem tissues were suspended in 3 mL medium and processed in a tissue homogenizer (Stomacher; Teckmar, Cincinnati, OH). The suspension was placed into 27 mL of prewarmed BSK-II + KR medium. The medium was incubated at 34°C for 5 weeks and examined at 1, 3, and 5 weeks by darkfield microscopy for the presence of live spirochetes.

Detection of Borrelia DNA in buffy-coat and tissue samples. PCR was used to detect *B. burgdorferi*–specific DNA in blood and tissue samples. To avoid contamination of the samples with previously amplified PCR products, we performed DNA extraction, amplification, and visualization in 3 physically separated rooms with different sets of equipment. To monitor for carry-over contamination, we distributed tubes containing water among tubes with tissue samples and handled all tubes the same way during DNA extraction and PCR. In addition, 25 tissue samples from an uninfected dog were extracted at the end of the test series with the equipment and reagents used for all other samples and subjected to PCR. Total DNA from buffy-coat samples collected at 2-week intervals throughout the experiment, from skin-punch biopsy samples collected at 4-week intervals, from 25 tissues collected during necropsy, and from cultured bacteria suspended in buffy-coat samples of uninfected dogs, which were used as standards, was extracted with the phenol/chloroform procedure [18].

One hundred microliters of buffy-coat samples or tissue samples were digested in a solution containing 100 μL proteinase K (1 mg/mL; Boehringer Mannheim, Germany), 150 μL of 1% sodium dodecyl sulfate solution (Sigma, St. Louis), and 75 μL of β-mercaptoethanol (Sigma) in a 1.5-mL microcentrifuge tube. Digestion was carried out under constant shaking for 2 h at 55°C (buffy coat samples) or 6 h at 55°C (skin biopsy and tissue samples). Subsequently, digests were transferred into phase-lock gel tubes (PLG I-H; Eppendorf 5 Prime→3 Prime, Inc., Boulder, CO) and mixed with 500 μL of 75% Tris-saturated phenol (pH 8; Sigma), and 25% chloroform/isoamyl alcohol (Fisher Scientific, Pittsburgh, PA). The organic phase was separated from the aqueous phase by centrifugation at 12,000 *g* for 5 min, and the gel contained in the tube formed a barrier between the 2 phases. After transfer of the aqueous phase into new PLG I-H tubes, extraction was repeated with 500 μL of 50% phenol and 50% chloroform/isoamyl alcohol and once again with 500 μL 100% chloroform/isoamyl alcohol. The recovered supernatant was mixed with 50 μL 3 *M* ammonium acetate (Sigma), and DNA was precipitated with 2 v of cold 100% ethanol. The recovered pellet was washed in 70% ethanol, dried under vacuum, and dissolved in 500 μL water. DNA yield and purity were determined with a spectrophotometer (Beckman, Fullerton, CA) at 2 wavelengths, $\lambda_1 = 260$ nm and $\lambda_2 = 280$ nm.

Five microliters of each DNA sample were used for PCR. *B. burgdorferi* DNA was measured semiquantitatively by amplifying the spirochete-specific *ospA* gene and comparing these signals to those of amplified canine actin, which was used to estimate the amount of total DNA per reaction tube. Primers previously shown to amplify a fragment of the *ospA* gene from different *B. burgdorferi* genospecies (SL1: 5′-AATAGGTCTAATAATAGCCTTAATAGC-3′ and SL2: 5′-CTAGTGTTTTGCCATCTTCTTTGAAAA-3′; [19]) were chosen for this study. Primers shown to amplify a fragment of canine actin cDNA (BAC-1: 5′-ATGTTCAGGGACTTTGGACG-3′, BAC-2: 5′-ACCAGCCATCCAGACAAAAC-3′; [5]) were chosen to detect the canine actin gene in this experiment. Reactions were carried out in 25-μL reaction volumes containing 1× PCR buffer II (Perkin Elmer, Foster City, CA), deox-

Table 2. Antibiotic treatment, episodes of lameness, and histopathological results at necropsy of dogs infected with *Borrelia burgdorferi.*

Dog no.	Antibiotic treatment	Episode of lameness, days after first TE, location							Histopathology
		1	2	3	4	5	6	After day 120	
A96-4/3	Azm	96, LF							NSL
A96-4/5	Azm	66, LF							Minimal NMA in R shoulder
A96-4/7	Azm	66, LF							NSL
A96-4/10	Azm								NSL
A96-4/8	Ctri	71, LF	85, LH	99, LF	101, LH	110			NSL
A96-4/9	Ctri	67, LF	80, LF	90, RF	102, LF	106, RF	120, LF+RF		NSL
A96-4/11	Ctri	83, LF	94, LF	102, RF	119, LF	121, LF+RF		205, LF; 280, LF	NSL
A96-4/12	Ctri								NSL
A96-4/1	Dox	100, LF+RF	104, LF						Mild nonsuppurative meningitis
A96-4/2	Dox	72, LF							NSL
A96-4/4	Dox	65, LF	84, RF						NSL
A96-4/6	Dox								NSL
A96-5/1	None	91, LF	106, RH						Moderate NPA; periarteritis
A96-5/2	None	50, LF						169, LF+LH; 434, PA[a]	Mild NPA; periarteritis
A96-5/3	None							123, LF; 431, PA[a]	Mild NPA; periarteritis
A96-5/4	None								Mild NPA; periarteritis

NOTE. Azm, azithromycin; Ctri, ceftriaxone; Dox, doxycycline; F, front; H, hind; L, left; NMA nonsuppurative monarthritis; NPA, nonsuppurative polyarthritis; NSL, no significant lesion; PA, polyarthritis; R, right; TE, tick exposure.

[a] Induced with prednisone treatment.

ynucleotide triphosphates (0.2 m*M* each; Perkin Elmer), primers (1 μM each), and Taq polymerase (0.63 U per tube; Perkin Elmer). The $MgCl_2$ concentration was adjusted to 2.2 m*M* and 2 m*M* for the *ospA* gene and actin gene amplification, respectively. PCR was performed in a GeneAmp 9600 PCR system (Perkin Elmer). The amplification reaction for the *ospA* gene consisted of an initial denaturation step (94°C, 2 min), 45 amplification cycles (94°C for 30 s; 60°C for 1 min; 72°C for 1 min), and a final 6-min 72°C extension step. Actin was amplified with a similar reaction protocol, except that only 30 amplification cycles (94°C for 1 min; 50°C for 1 min; 72°C for 1 min) were used. PCR products were separated on 1.5% agarose (Gibco BRL, Grand Island, NY) gels, stained with ethidium bromide (Sigma), and visualized over an ultraviolet light source.

Southern blot analysis was performed by use of a digoxigenin (DIG)–labeled probe and a DIG nucleic acid detection kit (Boehringer Mannheim). PCR products were separated on 1.5% agarose gels in 1× TBE-buffer and transferred overnight onto a nylon membrane (MSI, Westborough, MA) by capillary action. The membranes were then baked in a vacuum oven at 80°C for 2 h. A probe specific for the *ospA* gene (5′-AACAGCGTTTCAGTAGATTTGCCTGGTGA-3′) was labeled with DIG-ddUTP at the 3′ end with an end-labeling kit (Boehringer Mannheim). The membrane was prehybridized with the supplied blocking reagent at 68°C for 1 h and hybridized with a DIG-labeled probe at 54°C for at least 6 h. Bound DIG-labeled probes were detected with specific polyclonal antibodies conjugated with alkaline phosphatase and CSPD, a chemiluminescent substrate for alkaline phosphatase (Boehringer Mannheim). X-ray film (XAR; Kodak, Rochester, NY) was exposed for 1–5 min at room temperature with no intensifying screen.

Detection of DNA from heat-killed B. burgdorferi *organisms in skin-punch biopsy samples by PCR.* We wanted to determine how long DNA of dead *Borrelia* organisms remains in mammalian tissue and can be detected in skin samples of uninfected dogs. Low-passage *B. burgdorferi* organisms (strain N40; gift from Dr. Pachner, NJ) were grown in culture for 7 days to a density of 1.2×10^7 bacteria/mL. Spirochetes in the culture medium were killed at 65°C for 1 h. Twenty-five 5-μL aliquots of this spirochete suspension (1.5×10^6 organisms total) were injected intradermally into the skin of an anesthetized dog. Injection sites were located on the left rib cage of the dog and were 1 cm apart (4×4 cm area). Starting 1 day after inoculation, 10 4-mm skin-punch biopsy samples were taken while the animal was under local anesthesia from the area where spirochetes had been injected previously at weekly intervals. DNA extraction and PCR testing were carried out as outlined previously.

Results

Clinical signs. Despite the infection, dogs developed normally over the course of the experiment. After tick exposure, dogs showed only rare occurrences of elevated temperature (≥39.4°C [103°F]), which lasted for 1 day and on a single occasion for 2 days. Six of 33 episodes of lameness were associated with elevated temperature. After tick exposure, but before antibiotic treatment, 11 of 16 dogs developed clinically apparent arthritis in 1 or 2 limbs (table 2). The episodes of lameness lasted 3–6 days and resolved without treatment in all cases. The frequency of these episodes of joint inflammation varied among dogs with 1–6 episodes of lameness in the 11 clinically affected animals. Similar to observations we reported earlier [5], the first lameness episode occurred after a median incubation period of 71 days and was in the limb closest to the site of tick exposure in all dogs. All animals were infected on the left chest and the first episode of arthritis occurred in the left front limb. Subsequent bouts of lameness were observed in all extremities and were separated by 2- to 14-day intervals. After antibiotic therapy, only 1 ceftriaxone-treated dog (A96-4/11) showed 2 brief (1–2 days) episodes of lameness starting on day 205 and 280 after tick exposure, while 2 of 4 untreated control

dogs developed 1 lameness episode each on days 123 and 169 after tick exposure.

Antibiotic plasma levels. Over a 24-h period, plasma concentrations of azithromycin, ceftriaxone, and doxycycline were measured on day 3, 15, and 30 of treatment, and the results for all 12 treated dogs on day 30 are shown in figure 1. Azithromycin, which was given orally once a day, was detectable at a minimum concentration of 1.4 μg/mL plasma at 24 h. However, within 45 min of dosing, the plasma concentrations had climbed to a maximum of 6.1 μg/mL and then decreased gradually during the following 23 h. Plasma concentrations of azithromycin on day 3 of treatment were only ~60% of the concentrations detected on day 15 and 30 of treatment. As expected, intravenously delivered ceftriaxone accumulated rapidly in the circulation (up to 365 μg/mL) but was not detectable in the plasma by 6 h. Again, plasma levels on day 3 were lower and were only ~30%–50% of the concentrations detected on day 15 and day 30 of treatment. Doxycycline was given orally twice a day in 12-h intervals. Maximal plasma concentrations (5.5 μg/mL) were obtained within 1.5–3 h after treatment. At the end of the 12-h treatment cycle, between 1.3 and 4 μg of doxycycline per milliliter of plasma were still detectable. Doxycycline plasma levels did not differ on the 3 test days.

Antibody response. Antibody titers were evaluated at 2-week intervals. All dogs responded to the infection with *B. burgdorferi* and produced high antibody titers (400–500 KELA units) within 90 days of tick exposure (figure 2). Titers in the 4 untreated control dogs continued to increase slightly thereafter, and the dogs retained these high levels throughout the experiment (505–581 days after first tick exposure). All antibiotic-treated dogs, however, displayed a decrease in antibody titers, which began shortly before or after antibiotic treatment had been initiated. Azithromycin- and ceftriaxone-treated dogs displayed a steady persistent decline in antibody titers over the

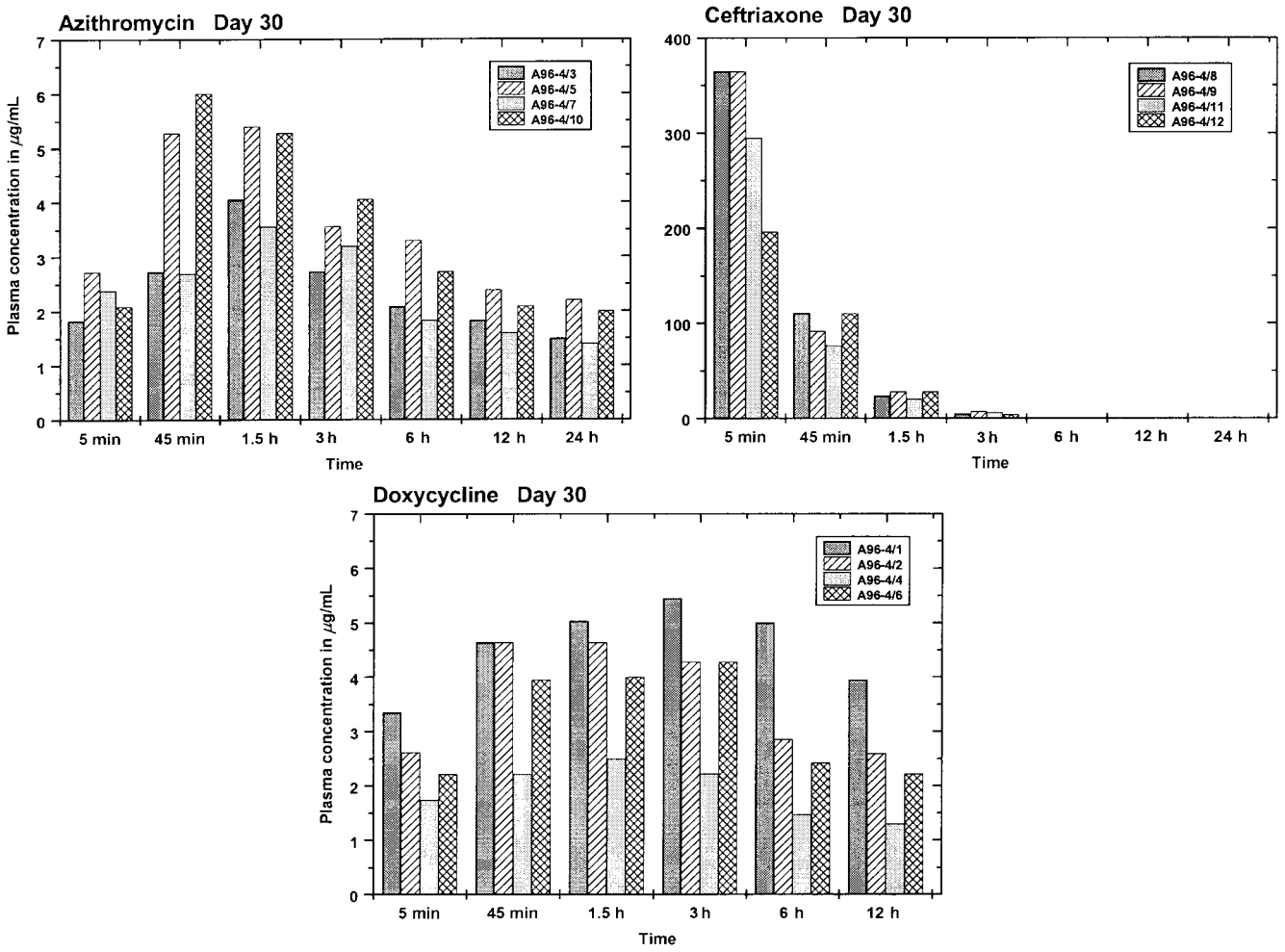

Figure 1. Blood plasma levels of the 3 antibiotics used in this study after dosing on day 30 of treatment. Sampling times are shown on the *x*-axis. Dogs received azithromycin orally at 24-h intervals, ceftriaxone intravenously at 24-h intervals, and doxycycline orally at 12-h intervals. Gel diffusion bioassays with antibiotic-specific organisms were used to measure blood plasma concentrations.

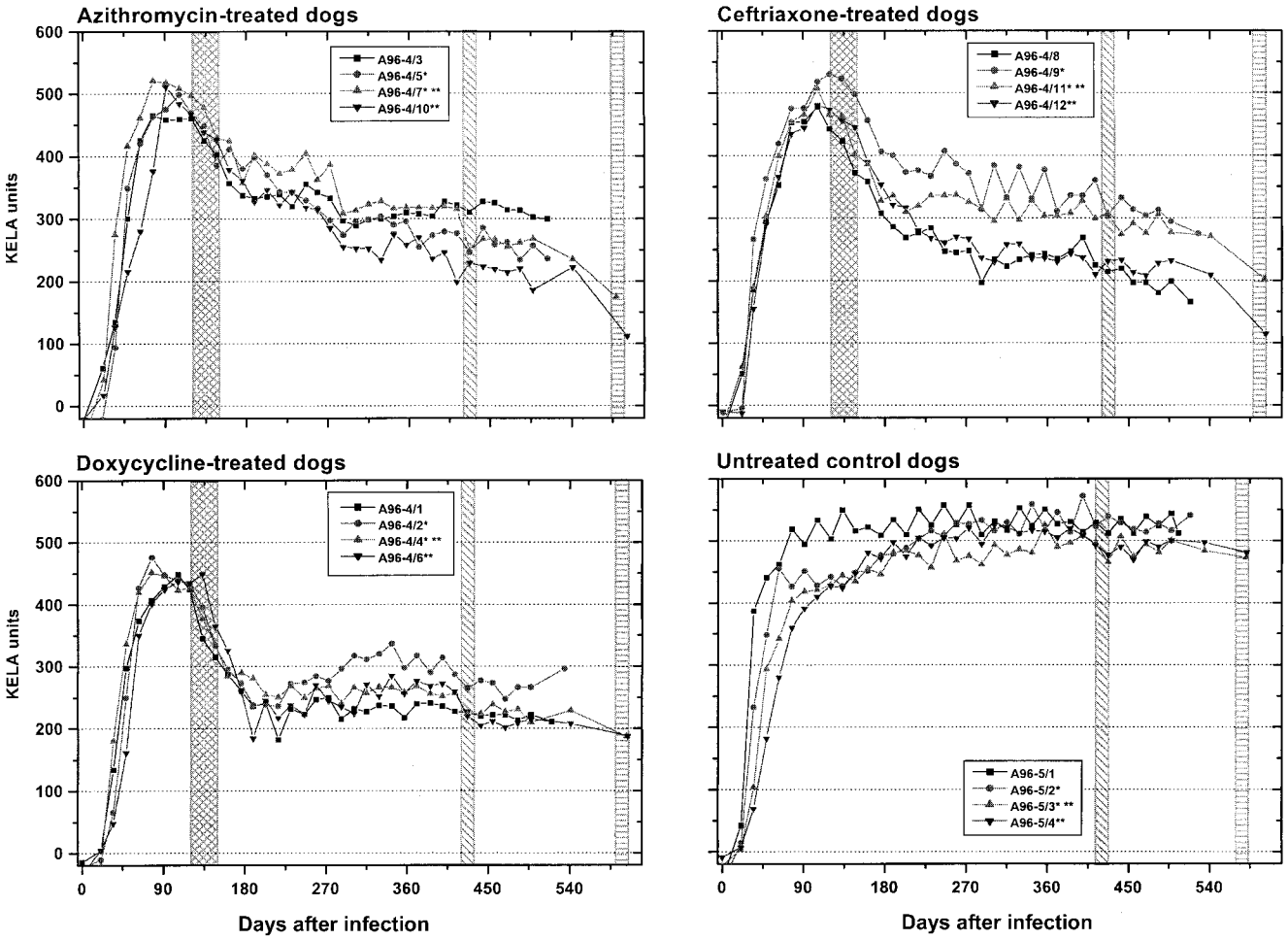

Figure 2. Kinetic enzyme-linked immunosorbent assay (KELA) antibody units to *Borrelia burgdorferi* in sera from dogs exposed to ticks on day 0. Treatment with antibiotics was initiated on day 120 and continued for 30 days *(crosshatched column)*. Time periods when prednisone was administered are indicated by columns with angled or horizontal marking. Prednisone-treated dogs are marked with asterisks; (*) dogs treated at day 420/413 after tick exposure, (**) dogs treated 14 days before euthanasia. Notice decline of antibody titers after all 3 antibiotics compared with untreated dogs. In contrast, prednisone did not appear to affect antibody levels.

study. In contrast, doxycycline-treated dogs responded to therapy with a marked decrease of antibody titers during treatment and for another 30 days after cessation of treatment. Antibody levels then plateaued, with constant or slightly rising antibody titers throughout the remaining observation period. Changes in antibody titers were also reflected in Western blots (figure 3). Untreated control dogs developed a specific pattern of bands typical for *B. burgdorferi* infection, which became more intense during the course of infection. The antibody response of antibiotic-treated dogs, however, was characterized by an intense infection-specific pattern up to 120 days after tick exposure (figure 3, lane 3), which correlated well with the peak antibody titers all dogs had developed at that time. After antibiotic treatment, many bands weakened or disappeared, but in some dogs a few specific bands remained present such as those at 23 kDa (OspC), 39 kDa, and 41 kDa.

Synovial fluids, CSF, and histopathology. At the time of necropsy, volumes of synovial fluids were recorded and aliquots examined for total and differential cell counts. All 3 synovial parameters of all 128 joints examined (8 large joints per dog) ranged within physiological limits. Also, CSF from all dogs did not show any abnormalities at the time of euthanasia. Postmortem tissues were fixed in buffered formalin, and for each dog a set of 20 hematoxylin and eosin-stained tissue samples (10 joints, 6 lymph nodes, pericardium, meninges, cerebrum, and cerebellum) were examined microscopically. Only 2 antibiotic-treated dogs showed inflammatory lesions, and they were minimal: in 1 joint (right shoulder, A96-4/5, azithromycin treated) and in the meninges (A96-4/1, doxycycline treated). Neither of these 2 dogs had received corticosteroids during the 2 weeks prior to necropsy, although A96-4/5 was dosed with prednisone 3 months earlier. In contrast, untreated control dogs

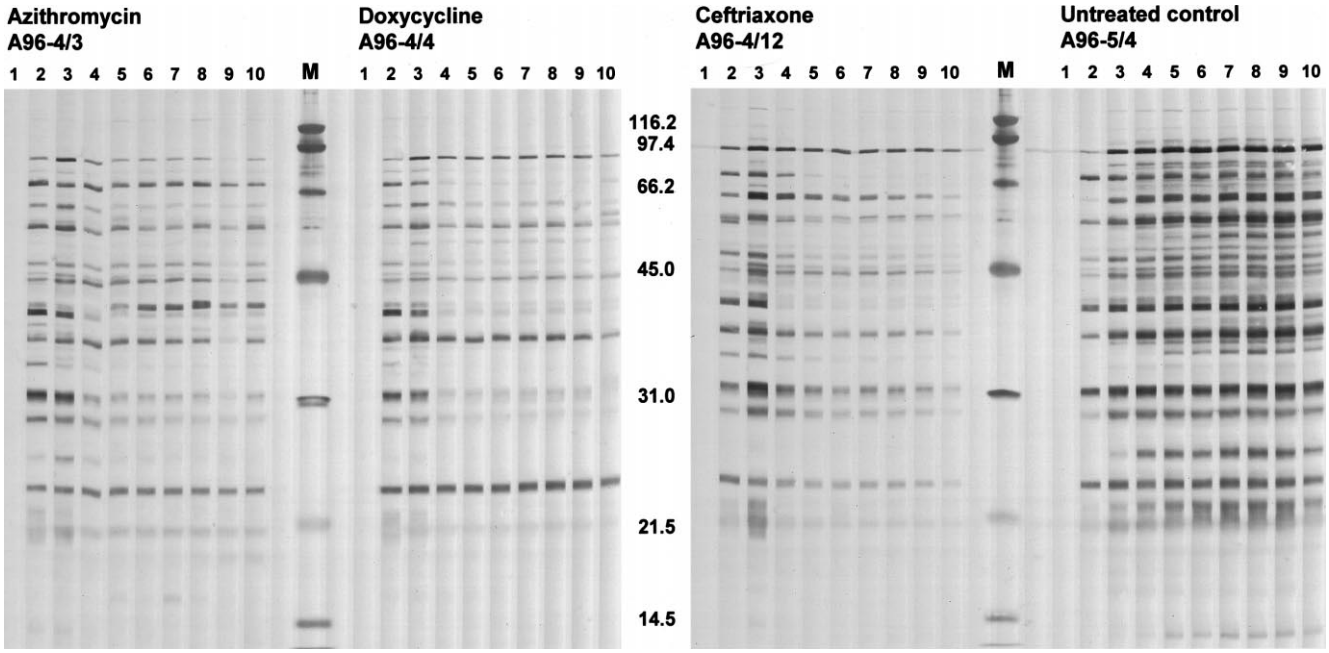

Figure 3. Western blot analysis of sera from 1 dog representing each treatment group at 8-week intervals. Lane 1, preimmune serum taken on day 0 of the experiment. The numbers in the center indicate molecular weights for corresponding bands of the molecular weight marker *(M)*. Note reduction in band intensity in the treated dogs after antibiotic administration (starting at *lane 3*) although some bands were not affected. In contrast, in the untreated control, bands persist and tend to become progressively more intense with time.

showed mild to moderate lesions in multiple joints at the time of necropsy, characterized by mononuclear cell infiltrates dominated by plasma cells below the synovial membrane. In addition, plasma cell infiltrations were observed in the tunica adventitia of arteries (periarteritis) located in the joint capsules of the tarsal joints from all untreated control dogs. The evaluated lymph nodes, which drained large joints and the area of tick attachment (left axillary and superficial cervical lymph nodes), appeared normal or showed mild to moderate cortical hyperplasia in untreated as well as in antibiotic-treated dogs.

Culture and PCR. Viable *B. burgdorferi* organisms or DNA were detected by culture and PCR, respectively, in 4-mm skin-punch biopsy samples collected at 4-week intervals and in a set of 25 tissue samples per dog collected during the necropsy. In addition, a set of 36 buffy-coat samples collected from each dog at 2-week intervals was subjected to PCR. Results are summarized in table 3. During the first 3 months after tick exposure, skin-punch biopsy samples were uniformly positive by culture and by PCR, with only 1 exception. By the fourth month, only 50% of the skin-punch biopsy samples were positive by culture, but 15 of 16 samples were still positive by PCR. The semiquantitative PCR technique showed clearly that 4 months after tick exposure the quantity of *B. burgdorferi*-specific DNA had decreased in these tissue samples (figure 4). After antibiotic treatment, none of the skin-punch biopsy samples and none of the postmortem tissue samples were positive by culture in dogs that had received azithromycin, ceftriaxone, or doxycycline. However, 9 (75%) of the antibiotic-treated dogs were positive by PCR in 1 or more tissue sample (skin or necropsy tissue). Interestingly, in all untreated control dogs, skin biopsy samples were sporadically positive by culture or PCR >4 months after tick exposure. At necropsy, 1 to 19 samples of a total of 25 tissue samples were positive by culture and 4–10 by PCR in the 4 control dogs. Testing over 500 individual buffy-coat samples by PCR was not very rewarding, with only 9 positive samples. Four dogs showed positive results, and the maximal number of positive samples was 3/36 samples collected in dog A96-4/4 and A96-4/10. Positivity of buffy-coat samples occurred at various times throughout the experiment.

Regular quality assessments of our test systems revealed that by culture we were able to detect a minimum of 1 high-passage spirochete per inoculum (data not shown), and 1–10 *B. burgdorferi* organisms per PCR reaction. Considering the fact that for our PCR reaction only 1% of the total amount of extracted DNA was used, this accounts statistically for ~100–1000 *B. burgdorferi* organisms per tissue sample subjected to PCR testing. Sequencing of the amplified PCR product and performing a Southern blot analysis with a *B. burgdorferi*–specific DNA probe confirmed also that the bands observed in the agarose gels were specific for this organism. Cross-contamination was monitored with test tubes containing DNA-free water distributed between the actual samples. These sham samples received the same treatment (DNA extraction, transfer, and dilution; PCR amplification) as did the tissue samples. In addition, tissue

Table 3. Results of assays for *B. burgdorferi* in skin biopsy, buffy-coat, and necropsy tissue samples from 12 infected dogs treated with antibiotics and in 4 infected untreated dogs.

		Skin-punch biopsy sample, culture/PCR[a]																	Buffy-coat samples, no. +[b]	PM tissue samples, no. +[c]
		Before AB treatment				During and after AB treatment														
Dog no.	AB	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17		
A96-4/3	Azm	+/+	+/+	+/+	−/+	−/−	−/−	−/−	−/+	−/−	−/−	−/−	−/−	−/−	−/−	−/−	−/−	−/−	0	0/1
A96-4/5	Azm	+/+	+/+	+/+	−/+	−/−	−/−	−/+	−/−	−/−	−/+	−/+	−/+	−/−	−/−	−/−	−/−	−/+	2	0/3
A96-4/7	Azm	+/+	+/+	+/+	−/+	−/−	−/−	−/−	−/−	−/−	−/−	−/−	−/−	−/−	−/−	−/−	−/−	−/−	0	0/0[d]
A96-4/10	Azm	+/+	+/+	+/+	−/+	−/−	−/−	−/−	−/−	−/−	−/+	−/−	−/−	−/−	−/−	−/−	−/−	−/+	3	0/2[d]
A96-4/8	Ctri	+/+	+/+	+/+	−/+	−/−	−/−	−/−	−/−	−/−	−/−	−/−	−/−	−/−	−/−	−/−	−/−	−/−	1	0/0
A96-4/9	Ctri	+/+	+/+	+/+	+/+	−/−	−/−	−/−	−/+	−/−	−/−	−/−	−/−	−/−	−/−	−/−	−/−	−/−	0	0/0
A96-4/11	Ctri	+/+	+/+	+/+	+/−	−/−	−/−	−/−	−/−	−/−	−/−	−/−	−/−	−/−	−/−	−/−	−/−	−/−	0	0/0[d]
A96-4/12	Ctri	+/+	+/+	+/+	+/+	−/−	−/−	−/−	−/+	−/−	−/−	−/−	−/−	−/−	−/−	−/−	−/−	−/+	0	0/0[d]
A96-4/1	Dox	+/+	+/+	−/+	−/+	−/−	−/−	−/−	−/−	−/−	−/−	−/−	−/−	−/−	−/−	−/+	−/−	−/−	0	0/2
A96-4/2	Dox	+/+	+/+	+/+	+/+	−/−	−/−	−/−	−/−	−/−	−/−	−/−	−/−	−/−	−/−	−/−	−/−	−/−	0	0/0
A96-4/4	Dox	+/+	+/+	+/+	−/+	−/−	−/−	−/−	−/−	−/+	−/−	−/−	−/−	−/+	−/−	−/−	−/−	−/−	3	0/0[d]
A96-4/6	Dox	+/+	+/+	+/+	+/+	−/−	−/−	−/−	−/−	−/−	−/−	−/−	−/+	−/−	−/−	−/−	−/−	−/−	0	0/1[d]
A96-5/1	None	+/+	+/+	+/+	+/+	−/+	+/−	−/−	−/−	−/−	−/−	−/−	−/−	−/−	−/−	−/−	−/−	−/−	0	14/4
A96-5/2	None	+/+	+/+	+/+	+/+	+/−	+/−	−/+	+/−	+/−	+/+	−/−	−/−	−/−	−/−	−/−	−/−	−/−	0	1/2
A96-5/3	None	+/+	+/+	+/+	+/+	−/−	+/−	−/−	−/−	−/−	−/−	−/−	−/−	−/−	−/−	−/−	+/−	−/−	0	10/4[d]
A96-5/4	None	+/+	+/+	+/+	−/+	−/+	−/−	+/+	−/−	−/−	+/+	−/−	−/−	−/−	−/−	−/−	−/−	−/−	0	19/10[d]

NOTE. AB, antibiotic; Azm, azithromycin; Ctri, ceftriaxone; Dox, doxycycline; +, positive; PCR, polymerase chain reaction analysis; PM, postmortem.

[a] Seventeen samples at 4-week intervals after tick exposure.

[b] Thirty-six samples collected at 2-week intervals after tick exposure and tested by PCR.

[c] Twenty-five tissue samples tested by culture/PCR.

[d] Fourteen-day oral prednisone treatment before necropsy.

samples from an uninfected dog were extracted and subjected to PCR amplification according to our standard procedure and with the same equipment. Both DNA-free water and DNA samples from an uninfected dog remained negative for *B. burgdorferi* nucleic acid.

Corticosteroid treatment. Of the 16 tick-infected dogs, episodes of lameness were not seen after approximately day 125 after infection with the exception of that which occurred in 2 dogs. It was of interest to determine whether immunosuppression by corticosteroids would reactivate clinical disease even after antibiotic therapy. Eight dogs (2 of each group) were dosed orally with prednisone for 14 consecutive days, starting 420 and 413 days after the first tick exposure in antibiotic-treated and untreated control dogs, respectively. Cortisol/prednisolone detection by ELISA on day 7 and 14 of the treatment demonstrated that 45 min after administration, cortisol/prednisolone levels in plasma increased above background by a factor of 4.3–17.7 (figure 5). During the 14-day treatment period, no clinical signs were observed, and, subsequently, all dogs previously treated with antibiotics remained free of clinical disease. However, both control dogs, which had not received antibiotics during the early phase of the study developed severe lameness 5 days (A96-5/3) and 7 days (A96-5/2) after the prednisone treatment had concluded. Pain and joint swelling was detectable in large joints of all 4 limbs. In dog A96-5/3, which showed the most severe signs of Lyme arthritis, recumbency because of lameness in all 4 limbs was accompanied by pyrexia for 2 days. Both dogs recovered without medical intervention within 7 and 5 days after the onset of lameness and did not show any additional signs of arthritis during the remaining time of the experiment. Figure 2 shows that antibody titers were not affected by corticosteroids during and after their administration.

To investigate whether immunosuppression immediately before necropsy would reactivate latent infection and so yield a higher percentage of positive tissue samples by culture or PCR, we dosed 8 dogs (2 from each group; 4 dogs previously treated with prednisone and 4 dogs which had not received prednisone previously) orally with prednisone for 14 consecutive days before euthanasia. None of the antibiotic-treated dogs became culture positive because of terminal prednisone treatment (table 3). In both dogs that had not received antibiotics during the trial, 10 and 19 tissue samples from a set of 25 were culture positive for *B. burgdorferi* at necropsy.

Detection of heat-killed organisms by PCR. To establish the period for which *Borrelia*-specific DNA can be detected in mammalian tissue using our probes, we injected 1.5×10^6 heat-killed, low-passage *B. burgdorferi* organisms (strain N40) into the skin of an uninfected beagle, and 10 skin-punch biopsy samples were taken at weekly intervals starting 1 day after injection. PCR analysis revealed that *B. burgdorferi* DNA was detectable up to 3 weeks after injection and not beyond this time.

Discussion

A review of the current literature leaves one with the impression that there is considerable controversy with respect to the treatment of Lyme borreliosis and its efficacy. Many different antibiotics are used during the early and late stages of the disease, may be given orally or intravenously, and it is

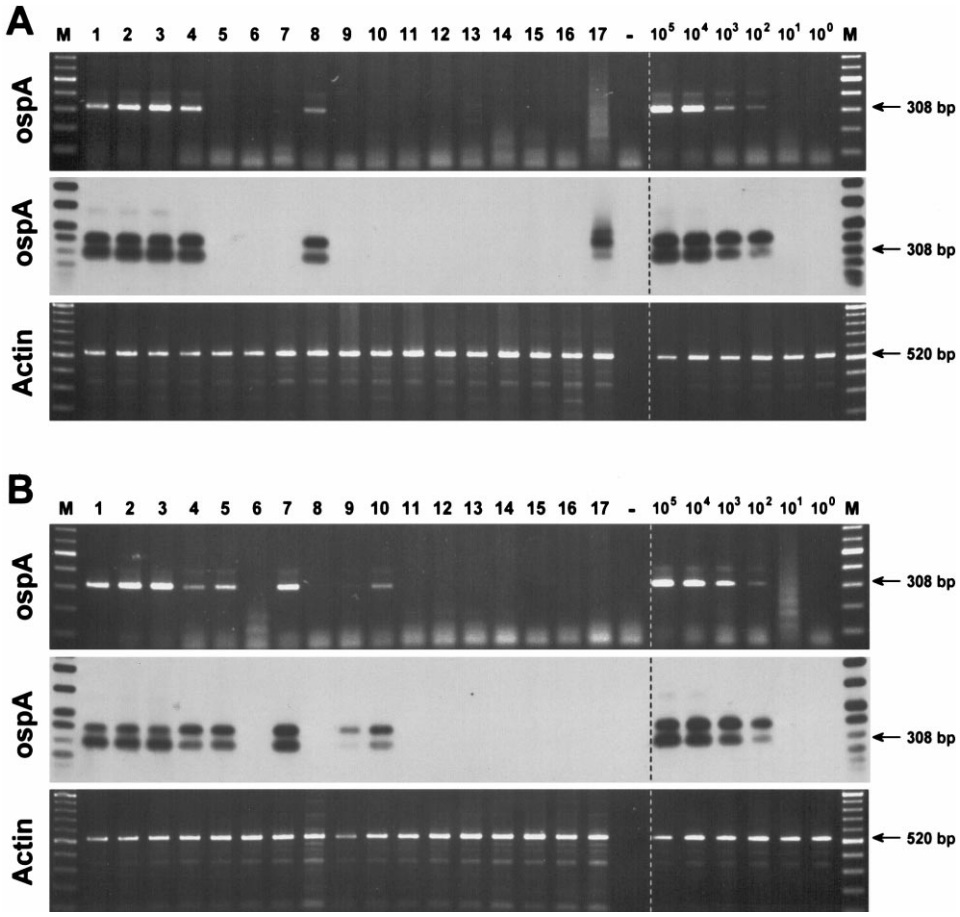

Figure 4. Semiquantitative detection of *Borrelia burgdorferi* DNA by polymerase chain reaction (PCR) by use of a specific primer set for the *ospA* gene in skin-punch biopsy samples taken at 4-week intervals from *(A)* a dog treated intravenously with ceftriaxone (A96-4/12) and *(B)* in an untreated control dog (A96-5/4). Lanes 1–4 show results before treatment was initiated in the antibiotic treated dog. *Lanes 5–17* show the sporadic detection of *B. burgdorferi* after antibiotic treatment and more frequent detection in the control. Specificity of the amplified DNA was verified by Southern blotting, which shows 2 amplicons of different size for *B. burgdorferi.* The right part of the blots shows a titration of *B. burgdorferi* prepared with buffy coats from uninfected dogs spiked with a known number of low-passage *Borrelia* organisms (strain N40); $1–10^5$ spirochetes were injected into 100 μL buffy coat cells. Sensitivity approached 100 spirochetes per 100 μL buffy-coat sample or 1 spirochete per PCR reaction, because only 1% of the total amount of extracted DNA was used per PCR reaction. The canine actin gene was used to assure satisfactory quality and constant quantity of the DNA tested in the experiment. -, water; M, molecular weight marker.

unclear whether the spirochetal organisms are eliminated by therapy. These questions prompted us to use our well-characterized dog model of acute Lyme arthritis [5, 15] to explore 3 different but relevant antibiotics, which are active either extra- and intracellularly (azithromycin and doxycycline) or only extracellularly (ceftriaxone), when administered either orally (azithromycin and doxycycline) or intravenously (ceftriaxone). We chose to treat our experimentally infected dogs for 1 month and at the dose outlined in the method section to guarantee that the animals received therapy for an adequate time period and dosage. The effects of the different treatment regimens were monitored comprehensively with a large number of samples and parameters to assure that many possible variables were evaluated.

Our laboratory has had considerable experience with experimental Lyme borreliosis in the beagle dog. After tick challenge, we see a highly consistent clinical outcome, namely 3–6 day-long episodes of mono- or oligoarthritis starting ~2 months after infection. These episodes are seen in ~75% of infected dogs with the remaining 25% infected but failing to show clinical arthritis. We have found that 2 cycles of tick challenge are more likely to induce clinical arthritis than is 1 cycle. After about the sixth month of infection, further episodes of clinical signs are not seen in most dogs, although the infection persists. Data from previous experiments [5] suggest to us that after skin challenge, the infection disseminates and the burden of infection progressively increases to some critical point at which joint inflammation is triggered. The first episodes of acute arthritis

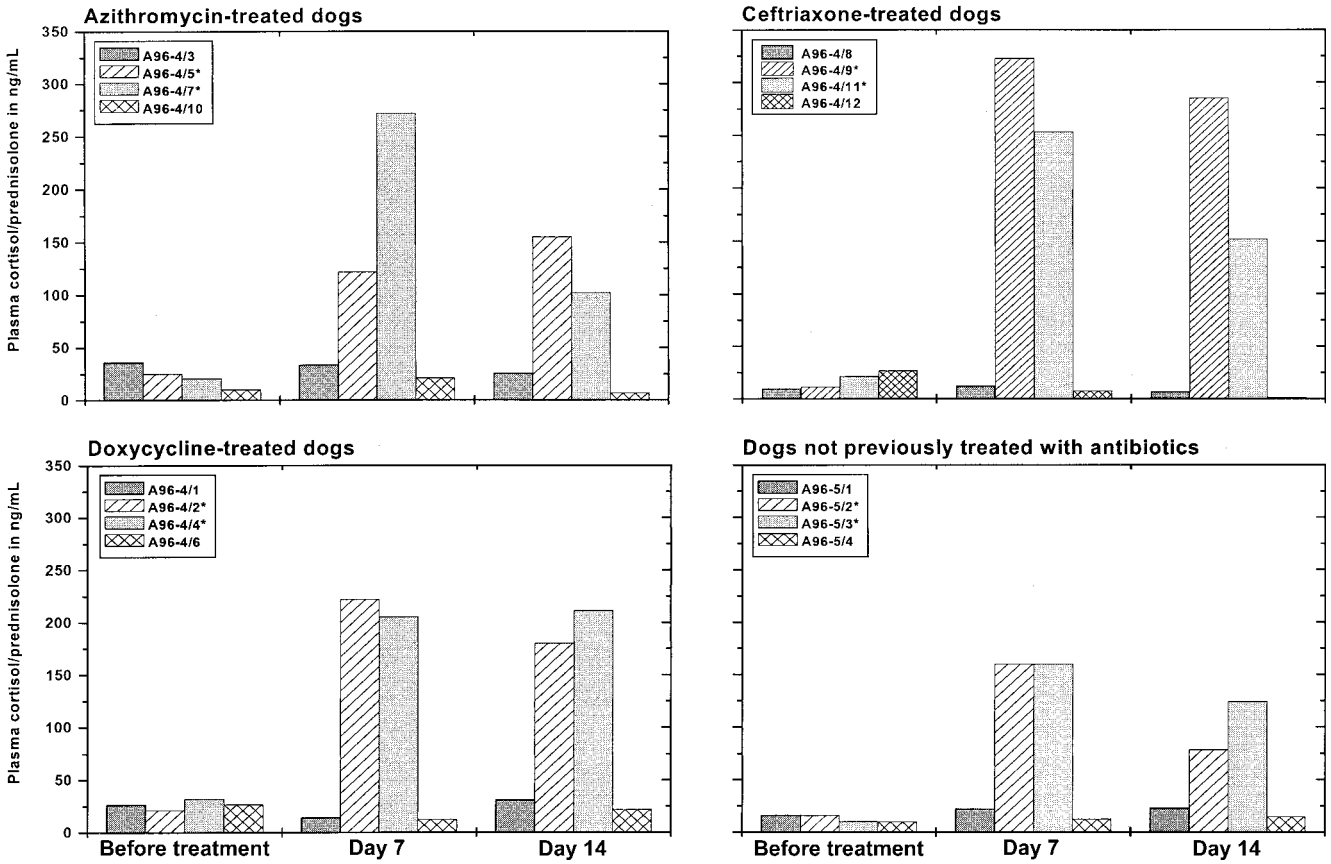

Figure 5. Cortisol/prednisolone levels in plasma samples from dogs treated with prednisone (*) for 14 consecutive days and untreated control dogs. Plasma was drawn before treatment and on the seventh and fourteenth day of treatment, 45 min after dosing orally with prednisone. Cortisol/prednisolone concentrations were measured with an ELISA by use of a cross-reactive antibody detecting canine cortisol and prednisolone.

are usually mono- or oligoarthritic, transitory, self-limiting, and probably induced by certain cytokines and chemokines such as interleukin-8 [5]. The absence of further episodes of arthritis after about the sixth month of infection may reflect the host's capacity to diminish the weight of infection to a low level insufficient to induce synovitis. This process may be evident in the 4 control dogs in this study (table 3, figure 4), in which sequential skin biopsy samples were positive by culture and PCR for the first 4 months after infection and then often gave negative results. However, these 4 dogs sustained high levels of specific antibodies against *B. burgdorferi* throughout the study and, at the conclusion, were found to be persistently infected. The apparent progressive decline in the burden of infection presumably occurs in response to the host's immunological reaction to the infection. The reactivation of severe polyarthritis in the 2 control dogs after a 14-day course of prednisone apparently reflects a disturbance of the balance between subclinical infection and immunity. It is noteworthy that at the time of corticosteroid treatment, these dogs had high titers of *B. burgdorferi*–specific antibodies, which did not change because of the corticosteroid treatment. It is also worth mentioning, that both dogs developed severe polyarthritis after prednisone treatment rather than mono- or oligoarthritis. These observations imply that during late infection, the cellular arm of the immune system is crucial for efficiently maintaining the *B. burgdorferi* infection in a subclinical state but that the organisms have previously disseminated throughout the body and are probably present in all joints. Specifically which elements of the host's immunity are responsible for maintaining the infection at a low level remains to be elucidated.

The data generated by this study indicate that at the time when disease manifestations are first appearing, antibiotic treatment of Lyme borreliosis has a long-term benefit for the patient. After all 3 antibiotic treatments, all but 1 dog showed no further clinical signs of spontaneous Lyme arthritis or after immunosuppressive therapy and at the time of necropsy showed only 1 minor joint lesion in 1 dog. However, antibiotic treatment, documented by high therapeutic concentrations in plasma, frequently did not eliminate *B. burgdorferi* from the body, although antibiotic-treated dogs differed from untreated control dogs regarding the form in which the spirochete could be detected in sequential skin biopsy samples and terminal post-

mortem tissues. In antibiotic-treated dogs, *B. burgdorferi* was detectable only by PCR and only in single-tissue samples, whereas in dogs that received no antibiotics, multiple-tissue samples taken at the time of necropsy were positive for *B. burgdorferi* by PCR and culture.

The detection of borrelial DNA but not viable spirochetes in dogs more than 360 days after antibiotic treatment is interesting and raises several questions. Do the data indicate an ongoing persistent infection in these animals or only the presence of DNA remnants of dead *Borrelia*; and secondly, are currently used culture techniques sensitive enough to detect *B. burgdorferi* long after antibiotic therapy? From this study and our previous investigations [20], it appears likely that *B. burgdorferi* maintains a persistent infection with live organisms albeit at a very low level. For example, KELA antibody titers soon fell in response to the antimicrobial drugs, presumably reflecting a progressively declining antigenic mass. Approximately 30–40 days after antibiotic therapy had ended, doxycycline-treated dogs showed constant intermediate antibody levels throughout the posttreatment time period, probably indicative of constant low-level antigen production and stimulation of the immune response. In contrast, the other antibiotic-treatment groups showed a constant steady, slow decline in antibody titers. In our studies published elsewhere [20], similar treatment regimens were initiated earlier, at days 50 and 70 after tick exposure. With these protocols, we were able to culture live organisms from 3 antibiotic-treated animals more than 70 and 170 days after therapy had ended. Interestingly, 75% of the animals in these early trials had achieved only low or intermediate antibody titers (150–300 KELA units) before antibiotic treatment was initiated, and, after treatment, antibody levels deceased to very low levels (<200 KELA units).

There seems to be a direct relation between the level of antibody titers in antibiotic-treated dogs and the success with which live organisms can be cultured from tissue samples. In our model, tissue samples collected at necropsy were more likely to be positive in culture when antibiotics abrogated the antibody response to *B. burgdorferi* early after infection. It can be asked whether reduced antibody levels after administration of antibiotics may reflect a more general decline in the host's immunological reactivity to the infection, which may even be detrimental in some chronically infected patients and perhaps explains the failure to eliminate the agent from the body.

Detection of microorganisms by PCR is limited by the fact that PCR does not distinguish between live and dead organisms. However, it seems very unlikely that DNA of nonviable organisms persists in mammalian tissue at detectable quantities for more than 360 days. As demonstrated by the injection of heat-killed *B. burgdorferi* organisms into the skin of an uninfected animal, DNA of dead organisms was detectable in our hands only for 3 weeks. These results are in concordance with a study in which persistent experimental infection with *Treponema pallidum,* the spirochetal agent of syphilis, was identified by PCR [21]. Wicher et al. discovered that DNA of dead *Treponema* organisms was removed from or degraded within rabbit tissue within 15-30 days after syringe inoculation. The possibility exists that our PCR data contained false-positive results. To avoid this potential problem, we performed a hazard analysis of all critical points of our procedure to limit and monitor DNA cross-contamination. Necropsy tissues of different dogs were collected on different days; DNA extraction, DNA amplification, and DNA analysis were performed in physically separated rooms; negative control samples containing DNA-free water were distributed among test samples; and tissue samples from an uninfected animals were included in the study. Furthermore, we did not use a nested-PCR protocol, a technique that is based on 2 rounds of PCR amplifications and is thought to boost sensitivity. The protocol of a nested PCR requires handling of already amplified product and therefore is a potent source of sample contamination.

The questions remain of how this bacterial organism possibly evades the combination of host immunity and antibiotic therapy, and what is the specific effect of the antibiotics we used on the spirochetes. The organism is susceptible to common antibiotics in vitro, especially to those used in this study, although the responsiveness to antibiotics varies among different strains of *B. burgdorferi* [22]. Patients with acute Lyme borreliosis are normally highly responsive to therapy and even chronic cases show a favorable response to antibiotic treatment [23]. However, patients may show relapses weeks to years after antibiotic therapy [24], which raises the question of reinfection or reactivation of the primary infection. It appears that *B. burgdorferi* can survive antibiotic therapy either by residing in privileged sites provided by the host or by using other strategies. *B. burgdorferi* is able to penetrate mammalian cells and can gain access to the intracellular space [25, 26], and there escape extracellularly active drugs. Alternatively, antibiotics may induce a change in their form from spiral to cystic bodies such that they are unable to actively proliferate in our culture system. Recently, Brorsen and Brorsen demonstrated that the spirochete can change its form in vitro [27]. The authors have shown by culture and electron microscopy that *B. burgdorferi* is able to produce cysts. These cysts survived unfavorable conditions for a long time and converted back into spiral-shaped organisms when transferred back into fresh medium. Interestingly, cystic forms were reported decades ago for other *Borrelia* species—*B. duttonii* and *B. vincenti* [28, 29] Therefore, we speculate that after antibiotic treatment, the infection persists at very low levels with viable organisms that cannot be rescued by our culture system and that fail to enter the proliferative phase, and so fail to induce clinical arthritis after immunosuppression of the infected patient. This may account for the positive PCR results in light of our evidence that DNA of dead spirochetes is detectable in tissue for no longer than 3 weeks.

Finally, this comprehensive study provides additional information that extends beyond the issue of antibiotic therapy. We

observed that PCR is not always superior to culture in detecting the presence of spirochetes. In persistently infected dogs, which did not receive antibiotic treatment during the experiment, culture was as sensitive as PCR. An explanation for this observation might be the sample size. Cultures of all our tissue samples were prepared by use of the complete tissue sample after homogenization in a Stomacher. In contrast, our PCR tests were prepared with only 1% of the total amount of DNA extracted from the same-size tissue sample. Consequently, it is not surprising that, especially during the late stage of infection when antibody levels were high and animals did not show clinical signs of Lyme borreliosis, tissue samples were likely to be positive in culture and not by PCR, considering a probable low level of infection with few organisms per tissue sample. Our studies show that at least in the dog, blood is an unreliable tissue to demonstrate *B. burgdorferi* infection. From a set of 36 buffy-coat samples per dog collected during the course of the experiment no more than 3 samples per dog were positive by PCR. PCR of blood samples can be inhibited by contaminants such as heparin [30], so we collected blood in EDTA-coated tubes and recovered DNA by chloroform/phenol extraction. Our titration produced with buffy coat cells of uninfected beagles inoculated with known numbers of *B. burgdorferi* organisms have shown that we were able to detect 1–10 spirochetes per PCR sample. Therefore, it is likely that only rarely were *Borrelia* present in blood samples taken sequentially during the course of the experiment. This would substantiate our hypothesis that *B. burgdorferi* does not use the circulatory system for dissemination; rather, the organism migrates actively through tissue [5].

In conclusion, the canine model of acute Lyme arthritis has provided further insight into this disease. We were able to investigate the status of the infection >360 days after antibiotic treatment and to collect data relevant to the chronic course of the disease seen in humans. We demonstrated that chronic silent infection with *B. burgdorferi* can be converted into active disease. Positive PCR results after therapy may reflect low-level persistent infection. Further research is needed to uncover the mechanisms that enable *B. burgdorferi* to gain a permanent foothold in the mammalian host.

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