Detection of Borrelia DNA in Circulating Monocytes as Evidence of Persistent Lyme Disease

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ABSTRACT

We report the detection of *Borrelia burgdorferi* DNA in circulating monocytes in a 31-year-old female who presented with a flu-like syndrome followed by neurological abnormalities after a trip to Southampton, Long Island, New York. ELISA and Western blot were negative. Lymphocyte proliferation assay to *Borrelia burgdorferi* was positive. *Borrelia burgdorferi* DNA was detected in circulating monocytes using a nested polymerase chain reaction (PCR). Treatment with parenteral ceftriaxone resulted in clinical improvement and repeat PCR on monocytes was negative. The use of detecting DNA by PCR from circulating monocytes may be useful in evaluating seronegative patients with a high suspicion of Lyme disease.

Key words: *Borrelia burgdorferi*, PCR, seronegative Lyme disease

INTRODUCTION

The diagnosis of Lyme disease requires positive serologic evidence of *Borrelia burgdorferi* infection in the appropriate clinical setting. Seronegative Lyme disease has been described[1] and frequently presents a diagnostic dilemma for the clinician. Alternative diagnostic tests include lymphocyte proliferation and antigen detection assays.[1-3] These methods have several drawbacks, including lack of standardization, as well as poor sensitivity and specificity. In this report, we describe a patient with neurological symptoms suggestive of multiple sclerosis who had evidence of *B burgdorferi* present in her circulating monocytes.

CASE REPORT

In 1993, immediately following a camping trip to Southampton, Long Island, a 31-year-old female resident of Nassau County, Long Island developed a mononucleosis-like syndrome with severe sore throat, neck tenderness, and profound fatigue. The sore throat resolved, but fatigue persisted. She began to have bilateral headaches, weakness on her left side, and difficulty walking. She also noted difficulty with concentration and word finding. A magnetic resonance imaging study done in September 1995 showed punctate linear areas of increased signal in the white matter, which were atypical for multiple sclerosis. She subsequently developed knee and ankle joint pain without swelling. She was diagnosed with depression in July 1994. A spinal tap in June 1996 revealed 7 white blood cells (WBC)/mm^2 (all mononuclear), protein of 53 mg/mL, glucose of 58 mg/mL, and 3 oligoclonal bands. Cerebral spinal fluid VDRL was negative. On physical examination in August 1996, she was afebrile and well appearing. Neurological examination revealed a left pronator drift and left upper extremity weakness.[1,3] She was unable to perform tandem walking and tended to drag her left foot. Finger-to-nose testing on the left side was abnormal. The remainder of the examination was within normal limits, including a rheumatological examination. Laboratory evaluation at this time revealed an antinuclear antibodies (ANA) titer of 1:80 (speckled) and an erythrocyte sedimentation rate of 32 mm/hr. VDRL was negative. Serological testing for Lyme disease showed negative ELISA and immunoblot assays in serum and cerebrospinal fluid. A lymphocyte

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proliferation assay to *B burgdoferi* performed by Specialty Laboratories (Santa Monica, CA) was markedly positive with a Lyme stimulated counts per minute (CPM) value of 18,741 and a Lyme stimulation index (SI) of 40.4 (stimulated CPM > 10,000 and Lyme SI >10 considered positive). A sonicated extraction of whole cell was used in the assay.

The patient received parenteral ceftriaxone for 6 weeks with improvement in her gait, left-sided strength, and resolution of the pronator drift. She also reported disappearance of all joint pain. A lumbar puncture at the end of treatment showed 1 WBC/mm^3, protein of 43 mg/mL, and glucose of 58 mg/mL with 7 oligoclonal bands detected.

MATERIALS AND METHODS

Monocytes were isolated and tested for *B burgdorferi* DNA as follows: 25 mL of whole blood from the patient, from 2 healthy donors, 5 osteoarthritis patients, and from a patient with multiple sclerosis were collected separately in heparinized tubes. The technologist was blinded to all clinical information. Whole blood was centrifuged over Ficoll-Hypaque medium (Organon Teknika, Durham, NC). A 5 mL suspension of the mononuclear cell layer was cultured in 25 cm^2 flasks. There were 200 µL of cell suspensions added to each chamber slide for 48-72 hours (RPMI 1640 medium with 10% fetal bovine serum, 5% CO_2 incubator at 37°C). Nonadherent cells (lymphocytes) and adherent cells (monocytes) were separated and washed.[4] Cells maintained in flasks were subjected to DNA isolation, and cells on chamber slides were stained by immunohistochemistry to identify cell populations. Slides were washed 3 times with PBS, then air-dried and fixed in 100% acetone for 3 minutes. The staining procedure was performed per manufacturer's instructions (Boehringer Mannheim Corporation, Indianapolis, IN) using mouse antihuman monocytes/CD 11b antibody. The majority (>90%) of adherent cells were confirmed to be monocytes (positive staining with mouse antihuman monocyte antibody), whereas <100% cells in the nonadherent fraction stained positive.

DNA was purified from each cell fraction with the Invitrogen Easy-DNA Kit (Invitrogen, San Diego, CA) per manufacturer's instructions. A nested PCR assay designed to optimize detection of different OspA genotypes was modified from Moter et al.[5] Briefly, external primers prZS7/3 1-1 and OspA-5 amplify a 662 base-pair (bp) fragment of the OspA gene, and internal primers OspA-6 and OspA-8 amplify a 392 bp fragment of the first product. The first PCR reaction mixture contained 30 µg total DNA, 1× PCR buffer, 1.5 mmol/L $MgCl_2$, 200 µmol/L dNTP, 0.25 µmol/L of each external primer and 1.5 U of Taq DNA polymerase (Perkin Elmer, Branchburg, NJ). The total volume was 50 µL. PCR amplification reaction was performed in a DNA thermal cycler (Perkin Elmer) under the following conditions: Denaturation at 94°C for 90 seconds, primer annealing at 45°C for 120 seconds and extension at 72°C for 120 seconds, for 30 cycles. After the first reaction, 5 µL of the reaction mixture was added to a new PCR mixture containing 1× PCR buffer, 1.5 mmol/L $MgCl_2$, 200 µmol/L dNTP, 0.25 µmol/L of each internal primer and 1.5 U of Taq DNA polymerase. Amplification conditions were changed to 35 cycles and annealing temperature 55°C. PCR products were analyzed on a 1.5% agarose gel. Ten fg of *B burgdorferi* B31 DNA served as a positive control. PCR amplification for *B burgdorferi* DNA has been used in our laboratory for several years without an incident of contamination. Four laboratories are used in a one-way-flow scheme so as to avoid contamination via re-amplification of amplicons. They included a dedicated room with dead-air hood for PCR set-up, room with thermal cycler, room with dedicated hood for second reaction set-up nested PCR, and a *dirty* room for analysis of products of amplification by ethidium bromide gel electrophoresis and Southern blotting. The laboratories used are in two different interconnected buildings, which allows us to completely separate the *clean set-up* and *dirty* areas for PCR.

To ensure that the DNA we isolated was amplifiable, 30 ng of cellular DNA was used to amplify exon 17 of the human amyloid precursor protein (APP) gene (primers, Research Genetics, Inc., Huntsville, AL).[6] Primers that amplify a region of the human papilloma virus (HPV 11) genome and HPV DNA were kindly donated by Dr. Bettie Steinberg, LIJ Department of Otolaryngology, to serve as a control for nonspecific amplification and were negative. PCR amplification conditions were 35 cycles, denaturation at 94°C for 60 seconds, annealing at 55°C for 120 seconds, and extension at 72°C for 120 seconds. As an additional control for the possibility of nonspecific amplification of unrelated bacterial DNA, a seminested PCR assay for amplification of *H influenzae*, *N meningitidis* and 16s eubacterial DNA was performed on the extracted DNA and was negative (data not shown).[7]

The gel was Southern blotted onto GeneScreen Plus nylon membrane (NEN Research Products, DuPont) with the use of standard conditions, then hybridized with a 25-bp probe (OspA-319, Nocton et al)[8] that was end labeled with fluorescein-11-dUTP (Amersham Life Science, Arlington Heights, IL). Chemiluminescent detection was performed with the ECL 3-oligolabelling and detection system (Amersham).

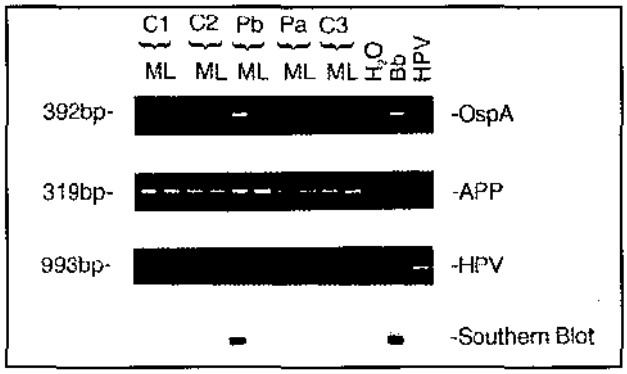

Figure. PCR detection of B burgdorferi *in human circulating monocytes.* ***Horizontal labels are DNA sources:*** *C1: healthy donor control 1; C2: healthy donor control 2; Pb: patient before treatment; Pa: patient after treatment; C3: multiple sclerosis control; M: monocytes; L: lymphocyte;* H_2O*: distilled water control;* Bb Borrelia burgdorferi *positive control; HPV: human papilloma virus.* ***Vertical labels are sizes in base pairs (bp) and names of amplification products:*** *OspA: outer surface protein A; APP: amyloid precursor protein; HPV: human papilloma virus.*

RESULTS

The PCR product of the APP gene was present on each sample except the human papilloma virus and *B burgdorferi* samples (see Figure). The patient's pretreatment sample showed a PCR product corresponding to the intended OspA target. The healthy donor controls, arthritis controls, (data not shown) and the multiple sclerosis controls were negative. Specificity of the product was confirmed by Southern blotting. The OspA product disappeared following treatment.

DISCUSSION

We present a patient with a presumptive diagnosis of multiple sclerosis whom, three years after the onset of symptoms, had evidence of *B burgdorferi* DNA in her circulating monocytes. Since there was a clinical response to antibiotic treatment for Lyme disease, which was accompanied by disappearance of *B burgdorferi* DNA, it is likely that the patient's signs and symptoms were because of, at least in part, chronic, seronegative *B burgdorferi* infection. Given the presence of oligoclonal bands in the CSF, it is also possible that the patient had concomitant multiple sclerosis and neuroborreliosis.

Seronegative Lyme disease has been described and been attributed in some cases to inadequate antibiotic treatment early in the infection.[1,9] While the incidence of seronegative Lyme disease is unknown, several patients in endemic areas present with arthritic and neurological signs and symptoms but lack serologic evidence of *B burgdorferi* infection, and therefore do not fulfill the CDC criteria for a diagnosis of Lyme disease. Certainly, the possibility exists that some patients have latent, chronic synovial or CNS infection that cannot be detected by commonly available tests.[8,10] Past reports have relied on lymphocyte proliferation assays for diagnosis, but critics have cited poor reproducibility, low specificity, and high cost. Recently, Mouritsen et al reported that *B burgdorferi* DNA can be detected in serum using a nested PCR method in seronegative patients.[11] A European patient with myositis, negative serologic, and lymphocyte stimulation tests for Lyme disease, but PCR evidence of *B burgdorferi* in his peripheral blood mononuclear cells was described.[12] The cell fraction was not specified, and the use of control specimens was not reported. Their patient had an atypical clinical picture but demonstrated a response to antibiotic therapy.

To the best of our knowledge, the present report is the first to demonstrate the presence of OspA gene segments from human monocytes. Monocytes including macrophages are highly active phagocytic cells that are present in both blood and tissues. Because antigens cannot directly activate lymphocytes, antigen-presenting cells, including macrophages, play a crucial role in the processing and presentation of antigens. The presence of *B burgdorferi* DNA in monocytes and the role of monocytes in killing have been described in vitro and in animal studies.[13-16] It is possible that the monocyte can serve as a haven for the spirochete. The life span of circulating monocytes is approximately 2 to 5 days prior to their entry into tissues, where they may persist for long periods. It has been shown that *B burgdorferi* is ingested rapidly by mouse monocytes and degraded in lysosomes.[14,16] There may be unknown host factors that can abort degradation, such as seen in HIV infection, where the virus may survive or proliferate in cells that are meant to eliminate it.[17,18]

A variety of physiologic abnormalities, including those of apoptosis,[19] glucocorticoid mediated monocyte function,[20] and regulation of nitric oxide synthetase activity[13,21] could cause monocytes to fail to present antigen to T-lymphocytes. Mouse monocytes can ingest pathogens without requiring prior opsonization with circulating antibody.[15] Thus, the appearance of an antibody response may be delayed or abrogated in some patients. Perhaps similar mechanisms are responsible for some cases of seronegativity.

We cannot exclude the possibility that the *B burgdorferi DNA* represented past infection and was unrelated to the patient's current symptoms. A beneficial response to placebo has been described in chronic fatigue syndrome patients with no documented infectious illness.[22] The disappearance of the PCR product, though, is suggestive that treatment resulted in clearance of the organism.

This assay may be helpful in cases where Lyme disease is high on the differential but serology is negative. The presence of DNA does not automatically imply active infection. Nevertheless, it may be one factor in the decision to administer a trial of antibiotics in a likely clinical setting after other diagnoses have been excluded. In addition, it may be useful to follow the efficacy of antibiotic treatment with the realization that the presence of DNA does not necessarily mean active infection.

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