Geographic clustering of an outer surface protein A mutant of *Borrelia burgdorferi*. Possible implications of multiple variants for Lyme disease persistence

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Abstract

DNA sequences encoding full-length outer surface protein (Osp) A were amplified from four joint fluid samples over 4.5 months from a patient with chronic Lyme arthritis, with a variant from wild type only found in sample 3. Rather than a mutation *in vivo*, these findings suggested a mixed infection in which *Borrelia* containing the wild-type and mutant *ospA* were waxing and waning in the patient's joint. If so, we reasoned that the mutant should be present in the community. We therefore took the novel epitope resulting from the mutation, expressed as a fusion protein in *Escherichia coli*, and performed Western blots on 80 high-titred stored sera; however, all except that of our index patient were negative. We then collected 36 stored sera from patients with Lyme disease residing within 10 miles of where the index patient had lived. An additional two sera from this circumscribed area were positive (*P* = 0.038). These findings show that results from single samples can be misleading, and suggest that the OspAs expressed in force late in Lyme arthritis are the same ones introduced initially into the host. Moreover, they allow a speculative mechanism for disease persistence not previously considered, in which antigenically distinct *B. burgdorferi* variant proteins present themselves serially to the immune system.

**Key words:** Lyme disease, *Borrelia burgdorferi*, Arthritis, Polymerase chain reaction, Outer surface protein A, Molecular epidemiology.

Lyme disease is caused by infection with the tick-transmitted spirochete *Borrelia burgdorferi* and is characterized by a transient rash [erythema (chronicum) migrans] and sometimes by subsequent neurological, cardiac, or joint involvement [1]. Most patients are readily cured at all stages by antibiotic therapy, but some individuals have persistent or recurrent disease [1]. Passive immunization with murine or human antibodies to outer surface protein A (OspA) can protect mice against *B. burgdorferi* [2], and active immunization with recombinant human OspA induces protective immunity [3, 4], but OspA antibodies elicited during natural infection in mice or men are unable to clear the spirochaete from the infected host. To examine antibody binding by OspA during the course of human infection, in earlier work [5] we amplified the operon encoding full-length *ospA* and *ospB* from synovial fluids of a patient with chronic Lyme arthritis—the first such recoveries from human material—at four separate time points over 4.5 months, and expressed OspA as a glutathione transferase (GT) fusion protein in *Escherichia coli* [5]. OspA monoclonal antibodies that passively protected mice from infection did not bind the expressed OspA from the third of those samples, due to a deletion in *ospA* that resulted in a frame shift and premature stop codon near the carboxyl terminus, where protective antibodies bind. Repeated amplification and sequencing, as well as the presence of patient antibodies to the expressed novel epitope, showed that this was not an artefact of the procedures employed.

Had we amplified only the third sample in that study, we might have concluded that the loss of protective epitope(s) due to *in vivo* mutation may be one reason that the spirochaete persists. However, the amplification of wild-type *ospA* from the first two samples from that patient, who already had had chronic arthritis for several months, makes this explanation less compelling. Moreover, the amplification of *ospA* without this deletion from a subsequent (fourth) sample of synovial fluid demonstrates that other mechanisms of persistence must be in play [5].

Ticks, mice, and humans have all been shown on
occasion to harbour more than one strain of *B. burgdorferi* in a single specimen [6–9]. We thought the most likely explanation for the current findings is a mixed infection in which *Borrelia* containing the wild-type and mutant *ospA* were waxing and waning in the patient’s successive joint fluid samples. If so, we reasoned that the mutant should be present in the community. To pursue this possibility, in the current study we performed Western blots on an array of sera from patients with Lyme disease, directed against the fusion protein containing the novel epitope.

**Methods**

**Patients**

The index patient, described in more detail elsewhere [5], was a young woman with a history of erythema migrans and an influenza-like syndrome, followed 10 months later by a swollen left knee, sore right temporomandibular joint, and high-titred specific IgG antibody against *B. burgdorferi* in her serum. Over several subsequent months, joint involvement persisted despite courses of oral and intravenous antibiotics, making available serial samples of synovial fluid for testing. One month after the fourth sample, an arthroscopic synovectomy produced an apparent cure; the patient is currently more than a decade without recurrence. Our knowledge of other individuals whose sera was stored at Yale University or obtained from colleagues elsewhere, was limited to test results and to towns of habitation.

**Immunoblots using the novel peptide**

The peptide corresponding to amino acids 232–254 of the *OspA* frame shift mutation region was generated and expressed in *E. coli* as a fusion protein with GT as described previously [5]. Lysates (1.5 μg) of *E. coli* expressing recombinant GT and GT-peptide were resolved by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) in a 15% polyacrylamide gel, transferred to nitrocellulose, and strips were probed with patient sera at a dilution of 1:500 in phosphate-buffered saline (PBS) with 1% bovine serum albumin (BSA) for > 3 h. Nitrocellulose strips were washed with Tris-buffered saline, pH 8, containing 0.2% Tween 20 (TBST), incubated with alkaline phosphatase-conjugated goat anti-human IgG (Promega, Madison, WI, USA), washed in TBST, and developed with 5-bromo-4-chloro-3-indoyl phosphate and nitroblue tetrazolium phosphate substrate (Kirkegaard and Perry Laboratories, Gaithersburg, MD, USA) as described previously [5].

For purification prior to testing (to suppress background binding to GT alone) selected patient sera were pre-adsorbed against recombinant GT bound to nitrocellulose. For pre-adsorption, 1 × 0.5 inch strips of nitrocellulose were incubated with 36 μg of GT (360 ng/μl in PBS), air-dried, and then each patient serum (at a 1:500 dilution) was incubated four times with GT strips (overnight at 4 °C and three times for 1 h at room temperature). Western blots were carried out simultaneously for adsorbed and the corresponding unadsorbed patient sera.  

**Statistical analysis**

Fisher’s exact test, two-tailed, was employed.

**Results**

In our initial description of the novel peptide, we reported that none of 30 other Lyme patients (early and late disease) or 10 normal human sera reacted with the novel epitope [5]. In the current study we performed Western blots to examine the reactivity to the novel peptide of 80 sera with titres by enzyme-linked immunosorbent assay (ELISA) ≥ 1600 (normal ≤ 200) against *B. burgdorferi*, 59 from patients with addresses in Connecticut. Any patient serum that reacted with the novel peptide was further analysed after that serum had been pre-adsorbed against GT alone, to rule out reactivity with GT rather than with the novel epitope. To our initial surprise, all these sera except that of the index patient were negative, or became so after adsorption of GT. We then considered the possibility that this particular variant may be rare and limited to ticks from a rather restricted area, and noted that only three of the 80 sera came from patients who resided within 10 miles of the geographic location where the index patient was living when she developed Lyme disease. We therefore sought from colleagues, stored positive sera from patients with Lyme disease who lived within that radius. From 36 such sera, we found two additional samples that reacted against the novel epitope, both of them from patients who resided within 8 miles of the index patient (Figs 1 and 2). (This region is highly endemic for Lyme disease, but because data on these two patients were limited to test results and to towns of habitation, we cannot say for certain in what town they were bitten.) The likelihood of this being a chance cluster is low (0 of 77 vs 3 of 40; *P* = 0.038).

**Discussion**

We discovered Lyme disease because of geographic clustering of patients with arthritis in the region of Lyme, Connecticut [10]. Now geographic clustering, this time of a *Borrelia* mutant, may be directing us further in understanding the workings of this illness. Here we make three points about this finding.

**In vivo constancy of OspA variants over time**

The expression of OspA declines rapidly in both the engorged tick and in the mammalian host [11, 12], and the early development of antibody to OspA is modest and transient; the return of OspA antibody production in force is a late finding, especially prominent with the development of Lyme arthritis [13–15]. The implication from the geographical data is that at least these two antigenically distinct OspAs, if they are indeed being re-expressed and are driving the antibody response to them in late disease, are isogenic with those initially
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introduced into the host. This finding is in keeping with work in C3H mice in which *B. burgdorferi* strains recovered after 1 yr of infection were isogenic with respect to their parent clone [16].

**Amplicons from single patient specimens may be misleading**

This point is partially discussed in the Introduction. We would further emphasize that the OspA wild type and the frame-shifted mutant may have been present to some extent in all the synovial fluid samples. Because polymerase chain reaction (PCR) amplification proceeds by geometric progression, the sequence that gets a head start, often because of its relative abundance or annealing efficiency, will quickly leave other sequences behind. Thus, other OspA mutants than the one expressed in sample 3 may also have been present in some samples, as well as variants of coding sequences not specifically sought here. Indeed, the recent establishment of the genomic sequence of the Lyme spirochaete reveals a large array of duplicated lipoprotein genes, unique to *Borrelia* spp. and of unknown function [17].

**A possible mechanism for chronic disease**

This is the first recovery, from the joint of a patient with Lyme arthritis, of DNA coding for more than a single antigenically distinct version of a given *B. burgdorferi* protein. Although OspA may not be the key protein in disease persistence, it may serve here as a model for those that are. It has been suggested that ticks and small mammals, thriving in terminal moraines in the Northern Hemisphere, may have been passing *Borrelia* (and other micro-organisms) back and forth for millennia [18], providing time for random mutation of an order not comparable to the spirochaete’s brief, dead-end sojourn in a human host. *Borrelia burgdorferi* is thought to find privileged sites in the mammalian host, where it is invisible to the immune system [1]. One such site may be inside cells [19–22]. Indeed, several intracellular spirochetes were recently described in synovial tissue from a patient with chronic Lyme arthritis [23]. Lyme disease is thought to result from the host response to a bacterium that is antigenically powerful but otherwise rather unaggressive [1]. In this formulation, in order for the inflammation to persist, *Borrelia*, or antigenic com-
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Fig. 2. Reactivity of patient sera to the novel peptide. The Western blot shows reactivity of patient and control sera with a novel peptide corresponding to amino acids 232-254 of an OspA frame shift mutation expressed as a GT fusion protein. (a) GT alone; (b) GT-peptide, which appears as a doublet. (A) Untreated sera. (B) A simultaneous immunoblot probed with the same sera after GT adsorption. In lane 5, GT alone (a) and the recombinant peptide (b) were probed with sera from the index patient; in lanes 3 and 4, with the two additional positive sera depicted in Fig. 1. Note that only GT alone was suppressed after GT adsorption (B: 3a, 4a, 5a); the recombinant peptide (B: 3b, 4b, 5b) retained specific activity. In contrast, in lane 1, probed with control serum, and in lane 2, probed with a representative high-titred serum from a patient with Lyme disease, both GT alone (1a, 2a) and the recombinant protein (1b, 2b) were suppressed after GT adsorption, indicating that the reactivity to both targets was due to GT. In lane 6, both antigens were stained with Coomassie brilliant blue to show that comparable amounts of protein were loaded per lane.

components of them, must periodically expose themselves to host defences. In the case of the mutant OspA, the loss of epitopes where protective antibodies bind [5] cannot by itself explain persistence of disease, because the patient did not clear wild-type OspA, either. However, one may speculate that in some patients with Lyme disease, the immune system must deal successively with mixed B. burgdorferi variants of the critical proteins involved in disease persistence, or with their antigenic products. These variants may leave privileged sites in forays that would keep the inflammation going, but which, by virtue of their differences, allow immune protection against earlier (and later) variants to subside. The natural subsidence of chronic Lyme arthritis—no one in one large series of patients had it for more than 4 yr [24]—is consistent with immune destruction of the last spirochaetal agonist, or with the eventual biodegradation of residual antigenic material.

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