

Detection of Multiple Reactive Protein Species by Immunoblotting after Recombinant Outer Surface Protein A Lyme Disease Vaccination

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Laboratory confirmation of the diagnosis of Lyme disease is based on the detection of an immune response to *Borrelia burgdorferi*. The serodiagnosis of *B. burgdorferi* infection is complex and may be further confounded by the immune response to the recombinant outer surface protein A (OspA) Lyme disease vaccine. To describe how the serological response to the recombinant OspA Lyme disease vaccine affects testing for antibody to *B. burgdorferi*, 240 specimens from 80 study subjects were obtained at defined intervals after recombinant OspA Lyme disease vaccination. Samples were tested by indirect enzyme-linked immunosorbent assay (ELISA), antibody capture enzyme immunoassay (EIA), and Western blotting (WB). After recombinant OspA Lyme disease vaccination, ELISA for 98% of the study subjects revealed reactivity. WB with use of OspA-containing *B. burgdorferi* strains as sources of antigens demonstrated multiple bands. Results of testing with a US Food and Drug Administration–approved WB kit showed homogeneous reactivity in the molecular weight region >30 kDa. Testing with OspA-free strains completely eliminated all vaccine-associated reactivity by both antibody capture EIA and WB.

Lyme disease is a multisystem inflammatory disease caused by infection with *Borrelia burgdorferi*. It is the most common vector-borne infectious disease in the United States. The diagnosis of Lyme disease depends on a combination of clinical features and laboratory demonstration of *B. burgdorferi* infection. Controversy exists surrounding the exact clinical spectrum of Lyme disease, on indications for test ordering, and on how to properly interpret test results.

Recently, recombinant outer surface protein A (OspA) vaccines have been proposed as a potential intervention to help control the Lyme disease epidemic. Results of 2 large pivotal phase III trials have recently been reported, demonstrating their safety and efficacy [1, 2]; final approval for the release of 1 of these vaccines was obtained in December 1998. Although many issues surrounding future vaccine use are unsettled, this vaccine is expected to be in widespread use in areas where Lyme disease

is endemic. However, preliminary observations have demonstrated that standard ELISAs for recipients of the purified recombinant OspA Lyme disease vaccine can show a positive serological profile [3, 4]. This circumstance has led to the suggestion that Western blotting (WB) might be the preferred test for evaluating possible *B. burgdorferi* infection in study subjects who have received the recombinant OspA Lyme disease vaccine [5]. We provide a detailed description of the serological profile after recombinant OspA Lyme disease vaccination; multiple test methodologies including WB were used in this analysis.

Subjects and methods

Study subjects were healthy at baseline and volunteered to participate in 1 of the 2 pivotal phase III trials [1, 2] of the recombinant OspA Lyme disease vaccine. None of our study subjects had a history of Lyme disease, except for 1 (lane 3 in figures 1 and 2) who was promptly treated for erythema migrans in 1992. No one contracted Lyme disease during this study. Descriptions of the vaccine trial entry criteria for these studies were reported in 1998 [1, 2]. A total of 240 specimens from 80 study subjects were collected. Specimens from 40 study subjects were collected at baseline and 30 days after the third dose of a licensed recombinant OspA Lyme vaccine (SmithKline Beecham Biologicals, Rixensart, Belgium). Specimens from an additional 40 study subjects were collected at baseline, 30 days after dose 2, 8 months after dose 3, and 24 months after dose 3.

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For collection of serum samples, there was separate institutional review board approval, and informed consent was obtained from the study subjects.

Guidelines for human experimentation of the US Department of Health and Human Services and/or those of the authors' institutions were followed in the conduct of the clinical research.

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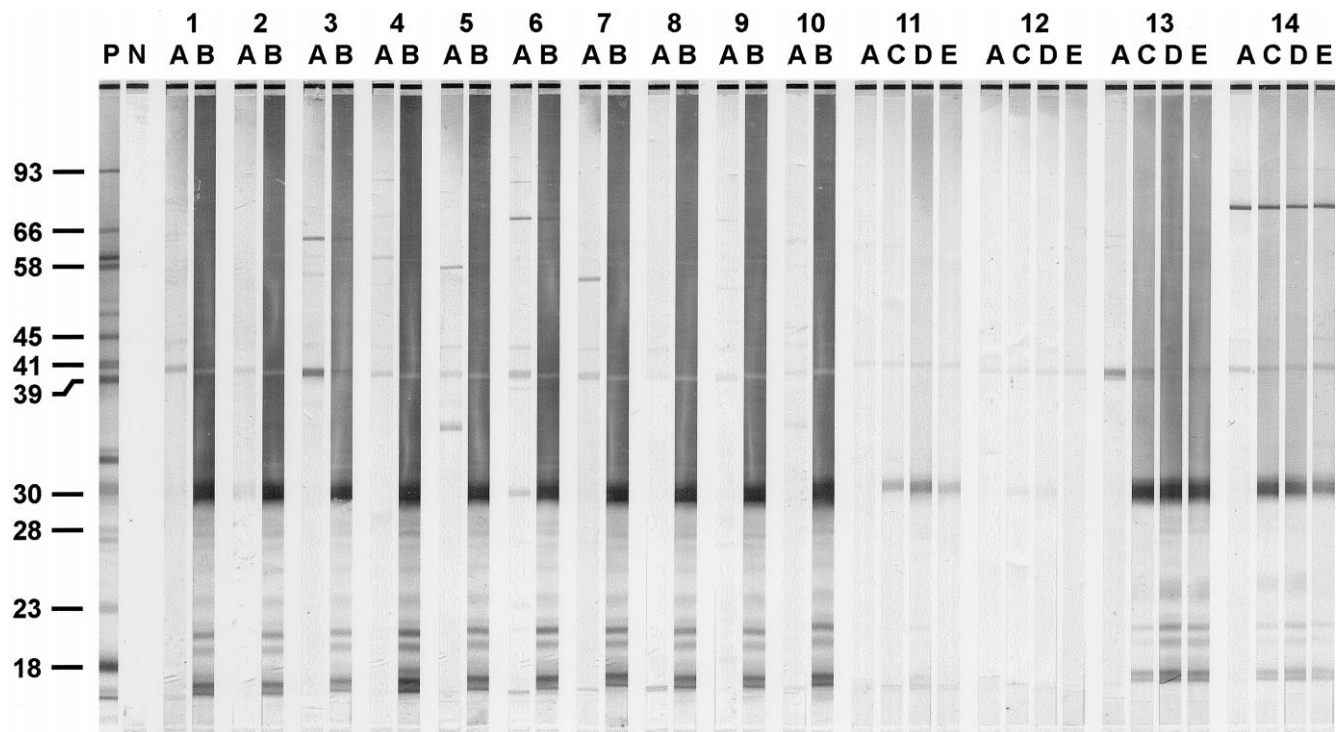


Figure 1. Results of Western blotting (MarBlot for detection of IgG antibody to *Borrelia burgdorferi*; MarDx, Carlsbad, CA) of serum specimens from 14 recipients of the recombinant outer surface protein A Lyme disease vaccine. Representative molecular weights (in kDa) are identified in the far left column. *P*, a positive control subject with PCR analysis–positive Lyme arthritis; *N*, a negative control subject. Study subjects 1–10: *A*, baseline; *B*, 30 days after dose 3. Study subjects 11–14: *A*, baseline; *C*, 30 days after dose 2; *D*, 8 months after dose 3; *E*, 24 months after dose 3.

tion (FDA)–approved ELISA kit (EIA for the detection of IgG and IgM antibodies to *B. burgdorferi*; MarDx, Carlsbad, CA) and a FDA-approved WB kit (MarBlot for detection of IgG antibody to *B. burgdorferi*; MarDx) were used for serological testing for Lyme disease. Testing and interpretation of test results were done according to the manufacturer’s recommendations. Antibody capture EIAs were performed and test results were interpreted according to previously reported methods [6], and specific IgM, IgG, and IgA isotypes were evaluated [7]. The test reagents used included standard native *B. burgdorferi* strains.

In-house WB was also performed as described elsewhere [8]. Briefly, the G39/40 strain of *B. burgdorferi* was transblotted onto 3-mm nitrocellulose strips at a final protein concentration of 26 ng. Antigen-bearing strips were incubated (37°C for 1 h) with 1-mL volumes of patient sera diluted 1:100 in PBS (pH 7.2) containing 0.3% polysorbate 20 and 5% nonfat dry milk. Patient IgG antibodies bound to *B. burgdorferi* proteins were detected with the substrate 5-bromo-4-chloro-3-indolyl phosphate nitroblue tetrazolium (Sigma, St. Louis) after incubation (37°C for 1 h) with alkaline phosphatase–conjugated goat antibody to human IgG (1:2000; Biosource International, Camarillo, CA). Antibody capture EIA and in-house WB were also

performed; genetically variant *B. burgdorferi* strains that lack the gene for OspA expression were used in these tests [4].

Results

Antibody capture EIA. A total of 240 specimens were tested by antibody capture EIA. Specimens from all 80 study subjects were negative at baseline. Of the 40 study subjects from whom a specimen was obtained 30 days after dose 3 of the recombinant OspA Lyme disease vaccine, 32 (80%) were seroreactive with ≥ 1 Ig isotypes, 5 (13%) were seroreactive with IgM, 30 (75%) were seroreactive with IgG, and 18 (45%) were seroreactive with IgA.

Specimens were obtained from the remaining 40 study subjects 30 days after dose 2. Of these 40 study subjects, 10 (25%) were seroreactive with IgM, 19 (48%) were seroreactive with IgG, and 8 (20%) were seroreactive with IgA. By 8 months after dose 3, 16 (40%) of these 40 study subjects remained seroreactive with IgG, 8 (20%) were seroreactive with IgA, and none were seroreactive with IgM. By 24 months after dose 3, 7 (18%) of these study subjects remained seroreactive with IgG, and none were seroreactive with IgA and IgM. Two years after dose 3, only IgG was detectable in 7 (18%) study subjects.

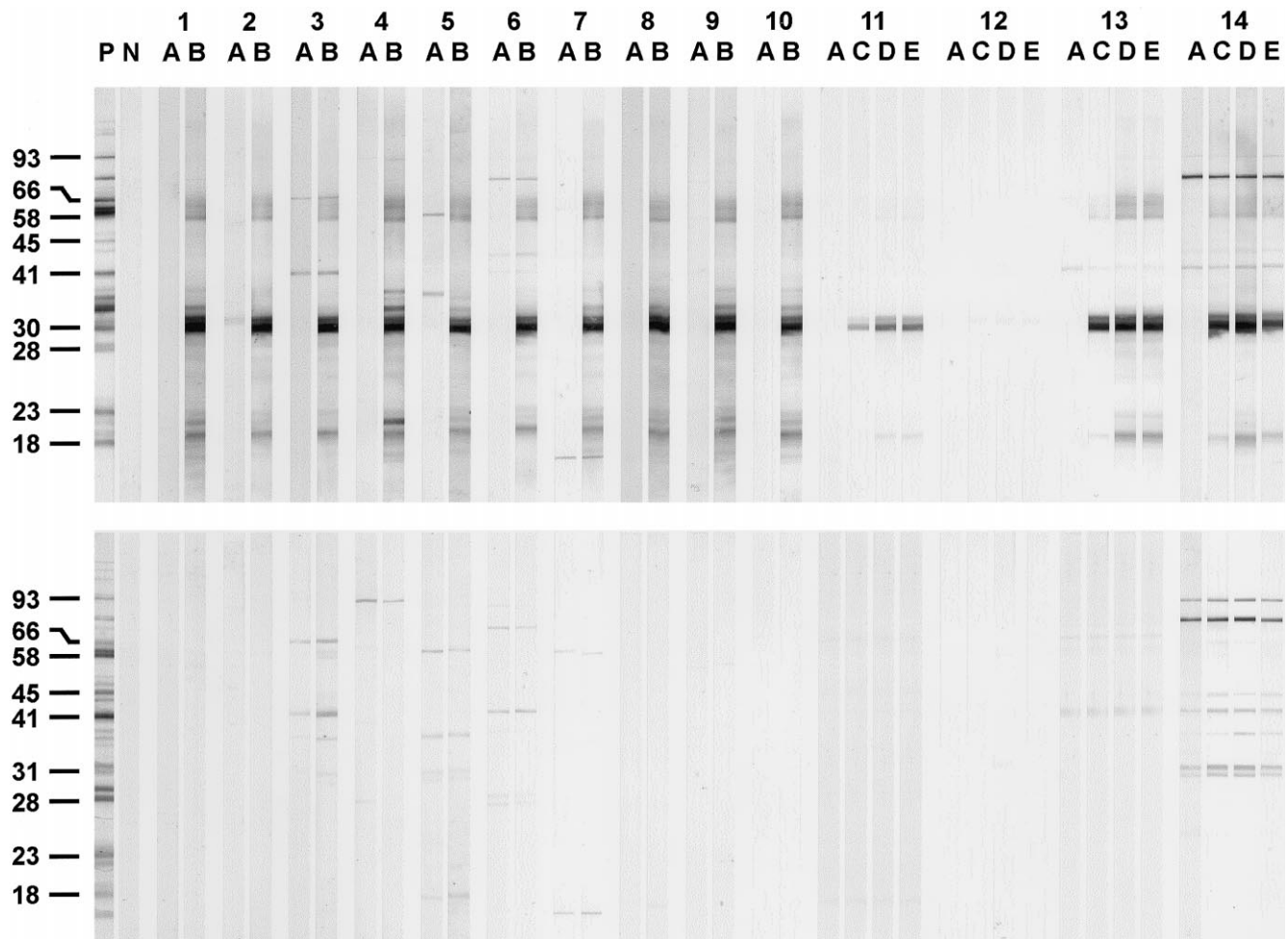


Figure 2. Results of an alternate methodology for Western blotting (WB) of serum specimens from the same 14 study subjects in figure 1 who received the recombinant outer surface protein A (OspA) Lyme disease vaccine. Top portion of the figure represents WB with use of a conventional *Borrelia burgdorferi* strain (G39/40), and bottom portion represents WB with use of a *B. burgdorferi* strain lacking OspA expression. Representative molecular weights (in kDa) are identified in the far left column. *P*, a positive control with PCR analysis–positive Lyme arthritis; *N*, a negative control. Study subjects 1–10: *A*, baseline; *B*, 30 days after dose 3. Study subjects 11–14: *A*, baseline; *C*, 30 days after dose 2; *D*, 8 months after dose 3; *E*, 24 months after dose 3.

When the 2 groups of study subjects were evaluated as a whole, IgM responses were most frequently observed 30 days after dose 2, IgG and IgA responses were most frequently seen after dose 3, and the response to all isotypes waned over time (table 1).

ELISA kit (for detection of IgM and IgG). Seropositivity was observed in 30 (97%) of 31 specimens obtained 30 days after dose 3 of the recombinant OspA Lyme disease vaccine. Among 93% of cases, values were found to be greater than for the high positive control subject for ELISA. The high positive control subject had a value of ~2.1 index units; the low positive control subject had a value of 1.2 index units. Specimens from vaccinated study subjects had a mean absorbance of 2.65, significantly higher than that of the high positive control. Results for representative samples of weak, moderate, and strong re-

sponders (identified by WB testing) at baseline, 30 days after dose 2, 8 months after dose 3, and 2 years after dose 3 are also shown in table 1. Although the numbers are small, the percentage of positive ELISA results was greatest 30 days after dose 3.

Commercial WB kit. Results of US FDA–approved WB testing were available for 10 study subjects selected to represent weak, medium, and strong responses. Specimens were available from each subject at baseline, 30 days after dose 2, and 8 and 24 months after dose 3. In addition, 16 study subjects from whom specimens were obtained at baseline and 30 days after dose 3 were tested. One of these study subjects (patient 3, figure 1) was positive at baseline (not the same subject who was positive by ELISA). After vaccination, multiple bands (or regions of serological reactivity) were seen in low molecular weight regions ≤ 30

Table 1. Results of antibody capture EIA and ELISA for study subjects who received outer surface protein A Lyme disease vaccine.

Serum collection	Antibody capture EIA			
	IgM	IgG	IgA	ELISA, polyvalent
30 d after dose 2	10/40 (25), 1.4 ^a	19/40 (48), 1.5 ^a	6/40 (15), 1.3 ^a	6/10 (60), 1.92 ^b
30 d after dose 3	5/40 (13), 1.6	30/40 (75), 6.1	18/40 (45), 2.1	30/31 (97), 2.65
8 mo after dose 3	0/40	16/40 (40), 1.7	8/40 (20), 1.1	7/10 (70), 2.05
2 y after dose 3	0/40	7/40 (18), 1.4	0/40	6/10 (60), 1.66

NOTE. Data are no. of vaccine study subjects with positive responses to *Borrelia burgdorferi* total no. tested (%), mean.

^a Mean geometric antibody response by antibody capture EIA of positive samples.

^b Mean absorbance by ELISA.

kDa—bands that were not present at baseline. In addition, an indistinct homogeneous region of blot reactivity above the molecular weight region of 30 kDa was observed. Virtually 100% of the specimens showed this type of reactivity 30 days after dose 3, and both multiple bands in the lower molecular weight regions and the homogeneous reactivity above the molecular weight region of 30 kDa continued 8 and 24 months after dose 3 in 5 (50%) of 10 specimens (figure 1).

In-house WB (with native B. burgdorferi strains). When the same specimens were tested with native *B. burgdorferi* strains by in-house WB, a different pattern of seroreactivity was observed. Most striking was the absence of the homogeneous nonspecific high molecular weight response following vaccination. Nonetheless, for many specimens, multiple discrete bands of reactivity were observed that were not present at baseline (figure 2). Characteristic bands of vaccine-associated reactivity included those at molecular weights of 16 (88% of specimens), 18 (70%), 20 (48%), 30 (98%), 32 (98%), 34 (58%), 38 (30%), 58 (88%), and 60 (78%) kDa. Reactivity at 18-, 20-, 30-, 41-, and 58-kDa bands was noted in some cases, which meet the Association of State and Territorial Public Health Laboratory Directors/Centers for Disease Control and Prevention (CDC) criteria for a positive WB that confirms the diagnosis of *B. burgdorferi* infection [9].

Antibody capture EIA and WB with use of OspA-free B. burgdorferi strains. When the same specimens were tested by antibody capture EIA, with use of the newly described strains of *B. burgdorferi* lacking native OspA expression as the test reagents, all were negative. (These were the same specimens that were 97% positive by commercial ELISA and 80% positive by antibody capture EIA.) When the same specimens were tested by WB with use of OspA-free strains of *B. burgdorferi*, all vaccine-associated reactivity was eliminated, specifically all of the multiple bands identified by WB following administration of the recombinant OspA Lyme disease vaccine. WB reactivity that was not revealed before vaccination was not shown by testing with OspA-free reagents (figure 2).

Discussion

Considerable attention has been paid recently to issues surrounding serological testing for *B. burgdorferi* infection [10].

Confusion and controversy exist regarding who to test, what type of tests to perform, and what interpretive criteria to use in different circumstances [11]. Guidelines have been proposed in these circumstances, to assist in avoiding the problems associated with overdiagnosis and/or misinterpretation of test results [12, 13]. This situation will become more challenging for study subjects who have received the recombinant OspA Lyme disease vaccine.

The present study was undertaken to further characterize the serological response to the recombinant OspA Lyme disease vaccine as determined by currently available serological tests. We focused on test methodologies, including antibody capture EIA and WB, with use of multiple *B. burgdorferi* strains (both OspA-containing and OspA-free native strains) as test reagents [4, 6, 7]. Since specimen quantities were available, representative study subjects were also tested by FDA-licensed ELISA and WB kits for descriptive and comparative purposes, making no claim or attempt to formally validate or invalidate these tests' performances for vaccinated study subjects.

The first significant finding of the study is that standard commercial ELISAs for detection of Lyme disease are seroreactive (i.e., "positive") for recipients of the recombinant OspA Lyme disease vaccine. The antigen reagents in these tests consist of OspA-containing native strains of *B. burgdorferi*. OspA is highly immunogenic, and when an immunocompetent host is challenged with single-protein recombinant OspA, antibodies to this recombinant OspA are formed. These antibodies will react with the native OspA from the ELISA test reagent, resulting in a positive test. Similar seroreactivity has been previously described by Zhang et al. [4]. We found similar results when postvaccination sera were tested by antibody capture EIA.

The second, but somewhat unexpected, finding is that immunoblotting of sera from vaccinated study subjects demonstrates a complex pattern of multiple bands. It had been assumed that immunoblot reactivity at the 30- to 31-kDa region represents antibodies to OspA and that vaccination with recombinant OspA would result in reactivity with only this protein. However, we clearly demonstrated reactivity at multiple band locations after vaccination with purified recombinant OspA.

When a commercially available FDA-approved immunoblot test kit was used, a substantial portion of the blot showed homogeneous reactivity in the molecular weight region above 30 kDa. This vaccine-induced reactivity was of variable intensity, could persist for many months after vaccination, and interfered with identification of any specific, discrete underlying bands in this area. When the same specimens were tested by an alternate immunoblot methodology developed in-house, homogeneous staining was not seen, but the occurrence of multiple bands was clearly seen. Reactivities at molecular weights of 16, 18, 20, 30, 32, 34, 40, 58, 60, and 65 kDa were frequent. Appreciation of the occurrence of this pattern of reactivity for vaccinated study subjects is crucial in the evaluation of possible *B. burgdorferi* infection in study subjects or populations who may have received the recombinant OspA Lyme disease vaccine.

Such a result, showing multiple reactive protein species (some of which are “CDC diagnostic bands”), might be misinterpreted as representing *B. burgdorferi* infection. Indeed, the authors (P. Molloy and V. Berardi) are aware of recipients of the recombinant OspA Lyme disease vaccine who had absolutely no clinical features of Lyme disease and were diagnosed with Lyme disease solely on the basis of these multiple bands (which were induced by vaccination; unpublished data). Even when investigators are familiar with this phenomenon, problems with interpretation of test results may arise in those situations when a vaccinated subject actually becomes infected with *B. burgdorferi* (i.e., a vaccine failure) or when other infections such as babesiosis or ehrlichiosis also present with flulike symptoms, giving the impression of vaccine failure. The challenge then will be to determine which bands result from recombinant OspA Lyme disease vaccination and which bands result from evolving *B. burgdorferi* infection. In the case of the FDA-approved immunoblot test kit, the identification of discrete bands at molecular weights >30 kDa is often unreliable or impossible because of the homogeneous staining in this area, compromising the ability of this test to diagnose Lyme disease in vaccinated study subjects. The manufacturer of the only currently FDA-approved (and released) recombinant OspA Lyme disease vaccine has suggested that vaccination does not interfere with serological evaluation of Lyme disease in vaccine recipients—a statement that is not supported by the data presented here.

The third significant finding is that immunoassays based on genetic variants of *B. burgdorferi*, which lack the gene encoding OspA, exist and can be used to resolve the problems associated with the vaccine response. If a reactive (i.e., “positive”) test is demonstrated by conventional ELISA or EIA and all of the reactivity disappears when there is a retest with OspA-free reagents (i.e., becomes “negative”), one can then conclude that the initial “positive” test is a reflection of recombinant OspA Lyme disease vaccination and not genuine exposure to *B. burgdorferi*. If the ELISA or EIA reactivity persists after testing with OspA-free reagents (i.e., remains “positive”), it may reflect true *B. burgdorferi* infection. Likewise, if multiple bands dem-

onstrated by conventional immunoblotting disappear when the serum is tested by an immunoblotting system with OspA-free reagents, then one can conclude that the observed bands were caused by a host immune response to the recombinant OspA Lyme disease vaccine and not *B. burgdorferi* infection. Persistence of multiple bands shown by immunoblotting with use of OspA-free reagents suggests that the reactivity represents genuine infection. However, to justify complete replacement of currently used antigens with OspA-negative reagents, additional studies will be required to demonstrate sensitivity equivalent to that of current tests.

Serum samples from 10 additional study subjects that were obtained 30 days after dose 3 of a second recombinant OspA Lyme disease vaccine (Pasteur Meriéux Connaught, Swiftwater, PA) were tested by the same methodologies (data not shown). Similar findings of multiple bands following vaccination were observed when tests with use of conventional OspA-containing strains were done but not when tests with use of OspA-free strains were done. This particular vaccine is not currently licensed.

Why purified recombinant OspA Lyme disease vaccination results in the observation of multiple bands on immunoblots has yet to be fully explained. One possible explanation is that there is sequence homology between recombinant OspA and other *Borrelia* antigens, such as outer surface protein B (some of which may be encoded by the same plasmid). Another possibility may be that laboratory preparation of immunoblot strips may result in degradation of recombinant OspA into smaller fragments (accounting for some of the lower molecular weight reactivity) and into dimers or trimers (accounting for some of the higher molecular weight reactivity). It is furthermore possible that there is some binding or association between OspA or its fragments and higher molecular weight *B. burgdorferi* proteins, accounting for some of the observed reactivity in this region.

One interesting implication of the finding of these specific multiple bands following recombinant OspA Lyme disease vaccination is that reactivity at many of the molecular weights proposed by the CDC for inclusion in criteria for specific confirmatory and diagnostic “bands” may represent an immune response to OspA. It is possible that reactivity to OspA is being “counted” several times. For example, reactivity at molecular weights of 18, 20, 30, and 58 kDa may all be “OspA bands,” although it is recognized that immune responses to other *B. burgdorferi* proteins may also result in reactivity at some of these same regions. Reactivity observed at the 58-kDa region may be to a 58-kDa *B. burgdorferi* protein or antigen or to an OspA epitope causing reactivity at 58 kDa, as these postvaccination findings have shown.

In summary, ELISA and EIA for detection of antibody to *B. burgdorferi* are positive by standard criteria for recipients of the recombinant OspA Lyme disease vaccine who do not have Lyme disease. When WB of serum samples from recipients of the re-

combinant OspA Lyme disease vaccine is performed with use of standard *B. burgdorferi* strains as test reagents, multiple bands are demonstrated on blots. These multiple bands potentially confound the interpretation of results of serological tests for vaccinated study subjects in contrast to statements made in the vaccine manufacturer's package insert. Both types of seroreactivity are eliminated if OspA-free strains are used as test reagents. These findings are of utmost importance in the interpretation of results of testing for Lyme disease in vaccinated populations. This serological finding will become a greater problem as these vaccines become widely used. Further validation of testing with these OspA-free strains in various stages of *B. burgdorferi* infection is under way.

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