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Effect of Immunization with Recombinant OspA on Serologic Tests for Lyme Borreliosis

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This study evaluated the effects of vaccination with OspA on the use of serologic tests as aids in the diagnosis of Lyme borreliosis. Sera from control and OspA-immunized mice and from OspA-immunized human volunteers were tested for serologic reactivity to *Borrelia burgdorferi*. Testing was performed with samples obtained prior to administration of vaccine and at 30 days following administration of an initial and a second dose of OspA vaccine. The assays used to assess serologic reactivity included an in-house-developed enzyme-linked immunosorbent assay (ELISA), an in-house-developed Western blot assay, two commercial Western blot tests, and a commercially available dot blot assay. Data obtained from this study demonstrate that immunization with the OspA vaccine will cause ELISA to yield positive results (as reported previously) for the majority of vaccine recipients. Results obtained from Western blot analysis indicate that vaccination with recombinant OspA induces production of antibodies which bind to several different borrelial proteins. The degree of reactivity detected by Western blotting varied greatly between the three assays used. The in-house assay showed the least reactivity, while one commercial Western blot test actually yielded positive test results for infection with *B. burgdorferi*. The usefulness of all three Western blot assays for the diagnosis of potential infection in a vaccine recipient is severely limited by the extensive reactivity caused by vaccination alone. Antibodies produced in response to OspA vaccination did not significantly affect the performance of the dot blot test; thus, it could provide a reliable means to test for infection with *B. burgdorferi* in OspA vaccine recipients.

Lyme borreliosis is a treatable bacterial infection transmitted to humans by bites from infected *Ixodes scapularis* ticks. Infection with the bacterium *Borrelia burgdorferi* has been reported in 48 states of the United States and is endemic within the temperate areas of Europe and Asia. Diagnosis of Lyme borreliosis rests upon finding either the typical rash, erythema migrans, or the combination of aseptic meningitis, cranial nerve disease, and flu-like symptoms or arthritis with the presence of detectable antibodies to *B. burgdorferi* (9).

Detection of antibodies to *B. burgdorferi* is most commonly accomplished by two methods: the enzyme linked immunosorbent assay (ELISA) and the Western blot test (WB). The ELISA uses soluble antigens of *B. burgdorferi* coated on polystyrene microwells. The antigen is usually prepared by sonication of the whole organism. This assay format allows inexpensive highly automated testing and is generally used for screening. In contrast, WB use electrophoretically separated components of the bacteria which are blotted onto a solid (usually nitrocellulose) substrate. This assay format allows identification of antibodies to individual components of the bacteria, yielding the potential for enhanced specificity; however, its performance and interpretation of the results are labor-intensive and it is comparatively expensive. There have been significant problems with the sensitivities and specificities of these assays, resulting in a Centers for Disease Control and Prevention (CDC) recommendation that ELISA be used for screening and WB be used for confirmation of the results for ELISA-positive specimens (2). Implementation of those recommendations may have improved the overall reliability of serologic tests for detection of infection with *B. burgdorferi*. U.S. Food and Drug Administration (FDA) approval of a vaccine for the prevention of Lyme borreliosis may cause a new confounding problem for the use of Lyme serology when vaccinated individuals are tested for infection. The approved vaccine, LYMErix, produced by SmithKline Beecham (SKB), uses a recombinant form of the OspA protein adsorbed on an aluminum hydroxide adjuvant (8). The native OspA protein of *B. burgdorferi* has a reported apparent molecular mass of 31 kDa on WB and is found in antigen preparations used for manufacture of ELISAs and WBs. The presence of this native OspA has been shown to be sufficient to cause ELISA positivity when sera from vaccinated individuals is tested (1).

It has been reported recently (1) that the immune response to the OspA vaccine induces antibodies that bind to several borrelial proteins, in addition to OspA. In that study, recipients of the Connaught Laboratories OspA vaccine were reported to have multiple low-molecular-mass bands present on WB, in addition to a broad dark band at an apparent molecular mass of 31 kDa. It was also reported that there was a general dark smearing in the high-molecular-mass regions of the WB for samples from recipients of each of the OspA vaccines (1).

This study was initiated to determine the extent of cross-reactivity resulting from vaccination with the FDA-approved SKB recombinant OspA vaccine and its potential impact on serologic tests. We examined sera from mice and 20 adult human volunteers who received two doses of the vaccine. Sera were tested by ELISA, WB, and a dot blot assay. The last test

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TABLE 1. ELISA results for mice[a]

Group	No. of mice	Mean SE ± OD	OD range
Healthy mice	3	0.131 ± 0.019	0.101–0.166
Vaccination one time	5	1.096 ± 0.097	0.712–1.218
Vaccination two times	5	1.421 ± 0.049	1.304–1.590

[a] Different groups of mice were tested for each time point, as serial bleeds from individual mice would not have provided sufficient serum for testing by all assays.

is composed of five different separated and purified or recombinant components of *B. burgdorferi*, only one of which contains OspA. Results indicate that cross-reactivity resulting from vaccination with OspA is more extensive than expected, essentially precluding the use of ELISA and severely limiting the usefulness of WB. The dot blot assay was found to be capable of distinguishing infection in vaccine recipients.

MATERIALS AND METHODS

Testing of the OspA vaccine with experimental animals was accomplished with three groups of female BALB/c mice. Group one, controls, received sterile phosphate-buffered saline (PBS) intraperitoneally. Groups two and three received one and two intraperitoneal injections of LYMErix vaccine (0.2 ml containing 12 μg of vaccine), respectively, at 15-day intervals. Blood was harvested and tested by an in-house ELISA, WB, and commercial dot blot assay by the same procedures described for samples from human volunteers, with the exception that antisera specific for mouse immunoglobulin G (IgG) and mouse IgM were substituted for the anti-human immunoglobulin antisera. The protocol was reviewed and approved by the A. I. duPont Hospital for Children's Animal Care and Use Committee.

Human vaccine recipients were selected from adult volunteers who were employees of the A. I. duPont Hospital for Children. Enrollment in the study required that volunteers complete a questionnaire detailing possible past exposure to *B. burgdorferi* and provide three blood samples (one at the baseline, one 30 days following administration of the first dose of LYMErix vaccine, and a sample 30 days following administration of the second dose of vaccine). None of the individuals enrolled in the study had a previous history of infection with *B. burgdorferi*. The study was reviewed and approved by the hospital's institutional review board for human studies.

ELISA. Harvested *B. burgdorferi* (strain ATCC B31) was sonicated in PBS on ice. A supernatant fraction obtained after centrifugation at 10,000 × *g* was collected, and the protein concentration was adjusted to 5 μg/ml. This antigen solution was incubated in microtiter wells for 2 at 37°C and was then fixed with 95% methanol and blocked with 2% bovine serum albumin (BSA) in PBS. Patient sera were diluted 1:80 in *Escherichia coli* adsorbent solution as reported previously (5). The diluted sera were incubated in the prepared microwells for 60 min at 37°C. Following washing, the wells were incubated with a 1:1,000 dilution of peroxidase-conjugated goat anti-human IgG (ICN Pharmaceuticals, Inc., Costa Mesa, Calif.) for 30 min at 37°C. After washing of the wells, 100 μl of 2,2'-azinobis (3-ethylbenzthiazoline sulfuric acid) substrate solution (Sigma) was added, and the plates were incubated for 10 min at room temperature. The wells were then read at 405 nm with a Titertek Multiscan instrument (Flow Laboratories). Titers were determined by comparison of optical densities (OD) to those on a standard curve. The negative threshold was less than 0.2 OD unit.

WB. For the in-house WB, *B. burgdorferi* low-passage strain (ATCC B31) grown in BSK-H medium (Sigma) at 29°C was harvested and resuspended in PBS. The spirochetes were diluted in sample buffer containing dithiothreitol and were electrophoresed in an 11% sodium dodecyl sulfate-polyacrylamide gel by a modification of the method described by Laemmli and Fayre (7). Separated antigens were transferred to a nitrocellulose membrane that was then blocked with BSA, dried, cut into strips, and stored in a desiccated form until it was needed. Sera were tested by WB for IgG and IgM antibodies by incubating the strips with a 1:100 dilution of test serum in PBS with 1% nonfat milk for 1 h at room temperature. The strips were washed and were then incubated with diluted biotinylated antisera (goat anti-human IgG at 1:1,000 or goat anti-human IgM at 1:500; Kirkegaard & Perry Laboratories, Gaithersburg, Md.) for 1 h at room temperature. The strips were again washed and incubated with a 1:1,000 dilution of peroxidase-conjugated streptavidin (Kirkegaard & Perry Laboratories) for 1 h. The strips were then washed and incubated with 4-chloro-1-naphthol substrate solution for 10 min. The reaction was stopped with distilled water, the strips were allowed to dry, and the reactivity was evaluated by comparison with controls.

Sera were tested with the QualiCode *B. burgdorferi* IgG Western Blot kit (Immunetics, Cambridge, Mass.) according to the manufacturer's instructions. Briefly, nitrocellulose membrane strips containing *B. burgdorferi* (low-passage culture of the ATCC B31 strain) were incubated with diluted patient sera for 30 min. After washing of the strips, alkaline phosphatase-conjugated anti-human IgG was added and the strips were incubated for 15 min. The strips were again washed and then incubated with alkaline phosphatase substrate (5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium [BCIP-NBT]) for 6 to 8 min. The reaction was stopped with distilled water, and the strips were dried. The reactivities of the test sera were determined by comparison with the weakly reactive control strip and the manufacturer's reference strip.

Sera were tested with the *B. burgdorferi* (IgG) Marblot Strip Test System (MarDx, Carlsbad, Calif.) according to the manufacturer's instructions. Briefly, diluted patient sera were incubated for 30 min with nitrocellulose strips containing electrophoretically separated antigens of *B. burgdorferi* ATCC B31. After washing of the strips, diluted alkaline phosphatase-conjugated anti-human IgG was added and the strips were incubated for 15 min. Following washing, of the strips, BCIP-NBT substrate was added for 4 to 12 min. The strips were washed with distilled water, dried, and compared to the weakly reactive control, the serum band locator, and the blot banding template to determine their reactivities.

Dot blotting. Sera were tested by the Borrelia DotBlot assay (GenBio, San Diego, Calif.) according to the manufacturer's instructions for running and interpretation of IgG and IgM antibodies. Briefly, diluted patient sera were reacted for 60 min at 55°C with test strips containing the following antigens: whole borrelia (low-passage) strain ATCC B31), HMW (analogous to p93), flagellin, p39, and OspC antigens. The dot blot strips were again washed in distilled water and soaked in an enhancer for 5 min. After another wash the strips were incubated in alkaline phosphatase-conjugated goat anti-human IgG or IgM for 30 min. The strips were again washed in distilled water and were then incubated in BCIP-NBT substrate for 5 min, rinsed, and allowed to dry. The strips were scored for their reactivities by comparison with the control.

RESULTS

Control and OspA-vaccinated mice were tested by ELISA for IgG and by WB and dot blot assay for IgG and IgM antibodies to *B. burgdorferi*. The results depicted in Table 1 show that a single OspA vaccination induced elevated levels of antibodies detectable by ELISA, with administration of a second dose causing a further increase in detectable antibody levels. The results obtained when sera from control and vaccinated mice were tested by WB (Fig. 1) paralleled those obtained by ELISAs. Following administration of the second dose of vaccine, mice produced IgG antibodies which bound to borrelial antigens with apparent molecular masses of 21, 23, and 28 kDa, in addition to the 31-kDa OspA antigen. Minimal IgM reactivity was observed by WB. When tested by dot blot assay, OspA-vaccinated mice were scored as negative for reactivity, with detectable binding only to the whole borrelial antigen observed (Fig. 2).

Sera collected from human volunteers at the baseline and 30 days after administration of the first and second doses of the vaccine were tested by ELISA and three different WB for reactivity to *B. burgdorferi*. The results of the ELISA presented in Table 2 show that all volunteers were negative at the baseline, and all but one were ELISA positive by 30 days following administration of the second dose of vaccine. Interestingly, only two volunteers seroconverted by 30 days following administration of a single dose of vaccine.

Results of testing by WB showed that none of the volunteers were positive at the baseline or following administration of a single dose of vaccine, although several showed detectable

FIG. 1. Selected WB strips from control and OspA-vaccinated mice tested for IgG antibodies to *B. burgdorferi*. The effects of one and two doses of OspA vaccine are shown for three mice from each group. Mice receiving two doses of vaccine had detectable IgG antibodies to several low-molecular-mass components. Reference markers for gauging apparent molecular masses are shown on the far right. Healthy mice (N) had no observable reactivity, while *B. burgdorferi*-infected mice (P) had antibodies which bound to several antigens, yielding a banding pattern similar to those observed in infected humans. Numbers on the right are in kilodaltons.

weak bands for IgG antibodies occurring on WB at the location corresponding to the 41-kDa flagellin antigen. However, by 30 days following the second administration of the OspA vaccine, all but one volunteer had multiple bands on WB when their sera were tested for IgG antibodies. None of the volunteers

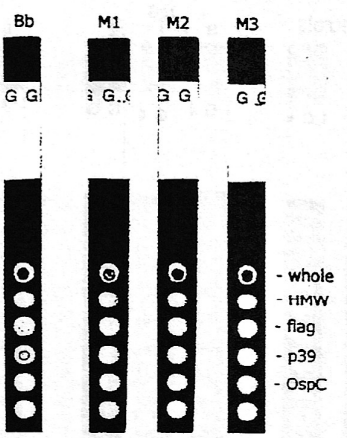

FIG. 2. Selected dot blot test strips for detection of IgG antibodies from a *B. burgdorferi*-infected mouse (Bb) and three mice which received two doses of OspA vaccine (M1 to M3) are shown. Vaccinated mice had detectable binding of IgG antibodies to the top dot (whole *B. burgdorferi* antigen preparation) only. The bottom dot serves as a reagent control, which reacts nonspecifically with human IgG. As expected, no reactivity to it was detected when mouse serum was assayed.

TABLE 2. ELISA results for volunteers[a]

Group	No. of volunteers	Mean SE ± OD	Range
Baseline	21	0.097 ± 0.006	0.047–0.146
Vaccination one time	21	0.122 ± 0.011	0.040–0.258
Vaccination two times	21	0.712 ± 0.079	0.087–1.622

[a] The ELISA used measured only IgG antibodies. None of the volunteers were positive at the baseline (cutoff, 0.2 OD unit). Two of the 21 volunteers were ELISA positive by 30 days following administration of the first dose of vaccine. One volunteer failed to seroconvert by 30 days following administration of the second dose of vaccine. That same individual was negative by all assays tested at all time points.

showed significant IgM antibody reactivity by WB at the time points at which serum specimens were collected. Representative WB strips from the in-house WB, commercial WBs (Immunetics and MarDx), and the dot blot assay are shown in Figures 3, 4, 5, and 6, respectively. On the strips from the in-house WB (Fig. 3), antibodies to the native OspA protein were detected as a broad (approximately 7-mm) dark band for all but one volunteer. Binding of IgG antibodies to several other components of *B. burgdorferi* was also observed at blot locations corresponding to apparent molecular masses of 17, 18, 21, 23, 25, 28, 30, 39, 41, 50, 58, and 60 kDa. The most frequent reactivity excluding that to OspA occurred at locations corresponding to the apparent molecular masses of 18, 23, and 28 kDa on the in-house WB. The results obtained indicate that the OspA vaccine causes more reactivity by the commercial WB than by the in-house assay. The WB from Immunetics (Fig. 4) was the most reactive, with sera from volunteers actually testing positive by the criteria of CDC/Dearborn (2). The MarDx WB (Fig. 5) was not as reactive, yielding bands at locations similar to those observed for the in-house WB. However, the high-molecular-mass regions of the MarDx WB (from 31 kDa up) showed a dark gray background which could cause problems for evaluation. The results

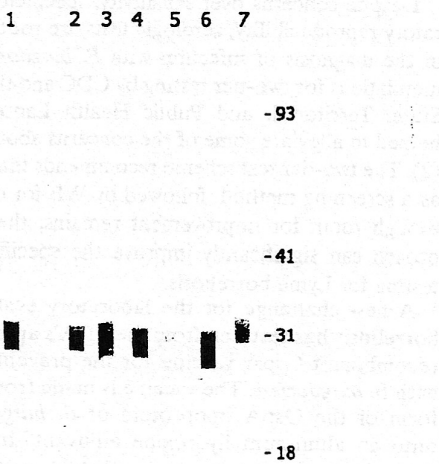

FIG. 3. Results of an in-house WB for IgG antibodies to *B. burgdorferi* in human sera. Strip 1, results for a pediatric vaccine recipient with suspected *B. burgdorferi* infection; strips 2 to 7, results for six volunteers from the vaccine study, respectively. Markers used to gauge apparent molecular masses (in kilodaltons) are shown on the far right.

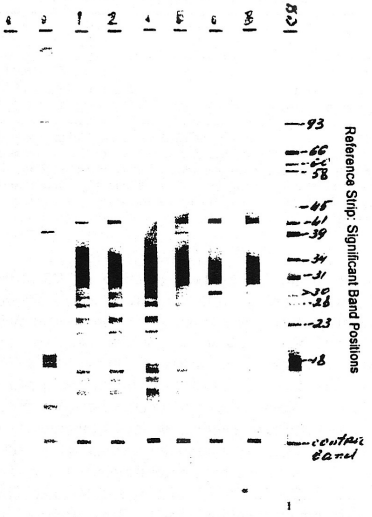

FIG. 4. Results of Immunetics WB with vaccine recipient serum for antibodies to *B. burgdorferi*. Arranged from left to right are weakly reactive control, positive control, strips from the six volunteers whose in-house WB strips were shown in Fig. 3, and the manufacturer's reference strip, respectively. Numbers on the right are in kilodaltons.

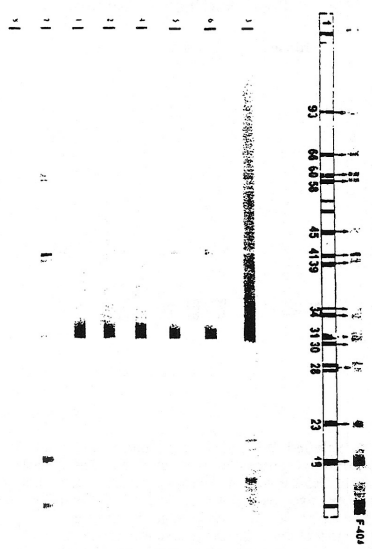

FIG. 5. Results of MarDx WB with vaccine recipient serum for IgG antibodies to *B. burgdorferi*. Arranged from left to right are the weakly reactive control, serum band locator, strips from the same six volunteers whose results are shown in Fig. 3 and 4, and the manufacturer's template, respectively.

of the dot blot assay (Fig. 6) showed that none of the volunteers became positive as a result of vaccination; however, one individual was positive at all time points (including the baseline).

DISCUSSION

Despite concerns over sensitivity, specificity, and interlaboratory reproducibility, serologic tests are routinely used to aid in the diagnosis of infection with *B. burgdorferi* (3). Recommendations for two-tier testing by CDC and the Association of State, Territorial, and Public Health Laboratory Directors helped to alleviate some of the concerns about Lyme serology (2). The two-tier test scheme recommends that ELISA be used as a screening method, followed by WB for confirmation. Although room for improvement remains, the use of this approach can significantly improve the specificity of serologic testing for Lyme borreliosis.

A new challenge for the laboratory evaluation of Lyme borreliosis has resulted from the FDA's approval of the SKB recombinant OspA vaccine for the prevention of infection with *B. burgdorferi*. The vaccine is made from a recombinant form of the OspA lipoprotein of *B. burgdorferi* adsorbed onto an aluminum hydroxide adjuvant. Information from the manufacturer and some published reports indicate that vaccination with the LYMErix vaccine can cause false-positive ELISA results and may result in the production of antibodies which cause bands on WBs at locations corresponding to the 31-kDa (native OspA) antigen and some lower-molecular-mass components (1). This study was initiated to determine the extent to which vaccinations with OspA would affect serologic test results and to examine an alternative test method for potential use with vaccinated patients. Owing to difficulties in obtaining well-classed and staged sera from individuals known to have been vaccinated

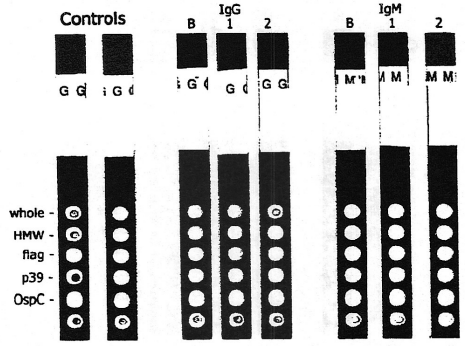

FIG. 6. Dot blot results for IgG and IgM antibodies for one of the volunteers. The two strips at the left are the positive and negative controls, respectively. The next three strips are IgG results for the baseline, after administration of one dose, of vaccine, and after administration of two doses of vaccine for a volunteer, respectively. The last three strips are the results for IgM antibodies for samples from the same three time points, respectively.

during clinical tests or on their own volition following FDA approval, we elected to use an animal model and human volunteers to conduct our assessment. The findings of the animal study showed that the LYMErix vaccine induced a high-titer antibody response following administration of the second dose of vaccine, which resulted in ELISA positivity and multiple bands on an in-house-developed WB. The reactivity on WB primarily involved IgG antibodies and was extensive enough to suggest that it could compromise the use of WB for diagnostic purposes. Subsequent testing with samples from human volunteers showed that their immune responses to vaccination evolved in a manner similar to that observed in our mouse model. Indeed, the extent of WB reactivity detected with human sera was in some instances greater than that observed with mouse sera and, thus, in our opinion, is likely to compromise the diagnostic usefulness of WB as well as that of ELISA for vaccine recipients. To ensure that this observed reactivity was not a fault with our in-house-developed assays, we tested a group of human sera using two commercially available, FDA-approved WBs. The results obtained by these commercial blots show that they detect even more reactivity in vaccinated individuals than the in-house WB. Indeed, the WB provided by Immunetics indicated that vaccine recipients will test positive by CDC/Dearborn interpretation criteria for WB, with multiple bands occurring in all molecular mass regions. The band locations scored on the Immunetics WB included 8 of the 10 bands denoted as significant by the recommended criteria (all except the 93- and 45-kDa bands). Results for the MarDx WB were apparently similar to those reported by Aguero-Rosenfeld et al. (1). In that study, sera from individuals vaccinated with the Connaught Laboratories OspA formulation (which is essentially the same as the SKB formulation but without the adjuvant) were found to have WB reactivity to some low-molecular-mass components of *B. burgdorferi*, in addition to the native OspA lipoprotein, and a darkening of the WB strip from below the location of OspA extending through all higher-molecular-mass regions. The authors stated that similar results were observed when sera from SKB vaccine recipients were tested (1). The findings of our study revealed that after administration of a second dose of vaccine individuals had detectable reactivity by the MarDx WB at the lower-molecular-size regions reported previously and also at regions corresponding to higher molecular sizes, including 39, 41, 58 to 60, and approximately 75 kDa. We also observed marked graying of the entire WB strips above the location corresponding to 31 kDa. We concur with Aguero-Rosenfeld et al. (1) that the presence of discrete low-molecular-size bands detected by all three WBs is probably a result of regions of amino acid sequence homology between different proteins and possibly of degraded OspA fragments (1). The extensive graying in the higher-molecular-size region of the MarDx WBs and the multiple discrete high-molecular-size bands observed by the Immunetics WB, neither of which was observed by the in-house WB, suggest that differences in WB manufacture is at least as important a factor in determining the extent of serologic reactivity resulting from OspA vaccination as the propensity of the vaccine to induce production of antibodies that cross-react with other components of *B. burgdorferi*. Regardless of the differences in the extent of WB reactivity resulting from the immune response to OspA vaccination, the utility of using any of the WBs to detect IgG antibodies to *B. burgdorferi* for diagnostic purposes is severely compromised. Testing for IgM antibodies by WB was not found to be significantly affected by OspA vaccination. However, since vaccination precludes the use of ELISA for screening, and IgM WB is recommended only for a limited time frame (4 weeks) and has a previously reported high rate of false positivity, we conclude that the standard two-tier test paradigm (ELISA followed by WB) is not reliable for diagnostic purposes for individuals known to have received the OspA vaccine (4, 6). Further work is needed to determine how to interpret the WB result when sera from vaccine recipients are tested. It may prove necessary to alter the current recommendations and determine the duration of the cross-reactive immune response to vaccinations following the third and possibly additional injections should they prove necessary.

As part of this study we investigated an alternative test method, the dot blot assay, which uses separated preparations of recombinant and purified components of *B. burgdorferi*. Our findings indicate that this test method is not compromised by vaccination with OspA. The test provides strips which hold physically isolated "dots" containing different antigen preparations. Only one dot, that containing whole borrelia antigen (analogous to the antigen preparation used in ELISA), became reactive as a result of vaccination. The other antigens, the high-molecular-mass, flagellin (p41), recombinant p39, and recombinant OspC (p23) antigens, were not affected by either IgM or IgG antibodies in the sera of vaccinees. In previous studies we compared the use of the dot blot assay with the MarDx WB and found that it performed essentially the same with respect to sensitivity and specificity for diagnostic purposes (6). The information on serologic status obtainable by WB is more extensive (essentially all immunodominant protein antigens are assayed simultaneously) than that which can be obtained by dot blot assay (whole antigen preparation and four purified or recombinant proteins), which makes WB more valuable for research or follow-up testing. However, the overwhelming majority of serologic tests performed to detect antibodies to *B. burgdorferi* are done so for routine diagnostic purposes. In this setting the dot blot assay performs comparably to WB and ELISA and, most importantly in the context of this study, is not affected by vaccination with the SKB LYMErix recombinant OspA vaccine.

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