Growth-Inhibiting Antibody Responses of Humans Vaccinated with Recombinant Outer Surface Protein A or Infected with *Borrelia burgdorferi* or Both

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Serial serum samples from a 2-year human trial of outer surface protein (Osp) A vaccine were analyzed by *Borrelia burgdorferi* growth-inhibition assay (GIA) and anti-OspA ELISA to assess the antibody responses of vaccine recipients and subjects with Lyme disease. Although 74% of OspA recipients had a reciprocal GIA titer $\geq 64$ after 3 vaccinations, none of the placebo recipients, even those with Lyme disease, had a GIA titer this high. The correlation between GIA and ELISA titers after 3 doses of vaccine was .84; however, more vaccine recipients had an elevated ELISA titer paired with low GIA titer than had a low ELISA titer with a high GIA titer. OspA-vaccine recipients who acquired Lyme disease had significantly lower serum GIA and ELISA titers after 3 immunizations than did age- and sex-matched OspA recipients without Lyme disease. Thus, vaccinated subjects had antibodies to native antigen on viable cells, and antibody assays with this specificity may predict protection of vaccinees against infection.

Lyme disease is caused by the spirochete *Borrelia burgdorferi* and is transmitted to humans by the bite of an infected tick [1]. A recombinant vaccine against *B. burgdorferi* infection is outer surface protein (Osp) A. This lipoprotein is expressed by the spirochete in culture in the laboratory [2], in the midgut of the tick [3, 4], and sometimes during late infection of humans [5]. Immunization with OspA confers protection against challenge with *B. burgdorferi* in mice [6–8]. When infected ticks feed on the immunized host, anti-OspA antibodies in the host’s blood kill or inhibit the growth of the spirochetes in the tick midgut [9, 10].

Recombinant OspA vaccines have been evaluated in humans by 2 phase III efficacy trials conducted in US areas where Lyme disease is endemic. In the first trial, subjects were immunized with OspA lipoprotein from *B. burgdorferi* B31 without adjuvant [11]. In the second trial, recombinant OspA lipoprotein from another strain was combined with alum [12]. For adults who received 3 immunizations at 0, 1, and 12 months, vaccine efficacy during the second year was 92% in the first trial and 76% in the second trial.

Experimental models of infection indicate that antibodies protect against *B. burgdorferi* delivered by syringe or by tick [9, 10, 13–16]. These findings suggest that a laboratory assay of antibody function could be used to predict protection against infection. Because laboratory-cultivated *B. burgdorferi* has an Osp protein phenotype that is similar to that in vector ticks [4], laboratory-reared spirochetes could be used as the target in the assay. In a preliminary study of 2 human volunteers immunized with OspA, we showed that their sera inhibited the growth of *B. burgdorferi* in vitro in a growth-inhibition assay (GIA) [17]. The GIA has been shown to provide an in vitro correlate of protection in the murine model of *B. burgdorferi* infection [6, 18–21]. A bactericidal assay for detection of an immune response after infection was reported, but the cells for the assay expressed another surface protein, OspC, instead of OspA [22].

The first goal of the present study was to use the GIA to characterize the antibody response of persons infected with *B. burgdorferi* and of persons with the clinical features of Lyme disease. The second goal was to evaluate the GIA as an in vitro correlate of protection with the use of sera from a phase III vaccine trial of OspA in an endemic area. For this last goal we also compared the GIA with a matrix-based ELISA with recombinant OspA.

Materials and Methods

*Bacterial strains and culture conditions.* *B. burgdorferi* B31 (ATCC 35210), the derivative isolate B314 [23], and *B. hermsii* HS1

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Informed consent was obtained from study volunteers. US Department of Health and Human Services and institutional guidelines were followed throughout the study.

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were grown in BSK II broth containing 6% vol/vol trace-hemolyzed rabbit serum (Omega Scientific, Tarzana, CA) and 100 μg/mL phosphomycin [24]. PAGE and immunoblot analysis with monoclonal antibodies confirmed that the B31 cells produced OspA and OspB but not OspC or OspD and that B314 cells produced OspC but not OspA, B, or D [23]. Cells were counted by use of a Petroff-Hauser chamber and phase-contrast microscopy. Spirochetes were stored in BSK II medium with 10% vol/vol dimethyl sulfoxide at −80°C.

Study population and sample selection. Subjects in the Pasteur Merieux Connaught OspA-vaccine trial were 10,305 healthy men and women, ≥18 years of age, from Connecticut, Massachusetts, New Jersey, New York, and Wisconsin [11]. Subjects were excluded if they had been taking antibiotics for an extended period or if they had impaired immune function or Lyme disease within 2 months before enrollment. The vaccine was 30 μg of recombinant strain B31 OspA lipoprotein that had been expressed in Escherichia coli; the placebo was diluent without OspA. All subjects received 2 doses 1 month apart at the beginning of the first Lyme disease season; 73% of subjects received a third dose 12 months after the first. Serum samples were drawn from subjects before the first immunization, 1 month after dose 2, immediately before dose 3, and 4–6 weeks after dose 3. Acute and convalescent blood samples were also drawn from subjects suspected of having Lyme disease. All sera were stored at −20°C. Lyme disease was defined as the presence of erythema migrans or the clinical manifestations of disseminated infection or both. Clinical manifestations consisted of seroconversion demonstrated by immunoblot analysis or isolation of B. burgdorferi from a skin biopsy [11]. One group of sera in the study was from the 80 vaccine or placebo recipients who had Lyme disease during the course of the trial. A second group of sera was obtained from a randomly selected group of 134 vaccine recipients and 67 placebo recipients who did not have Lyme disease during the trial and whose preimmunization sera did not inhibit B. burgdorferi. Of the 134 OspA recipients, 83 were male, and the mean age was 52 years. The numbers of subjects in age groups 18–29, 30–39, 40–49, 50–59, 60–69, and 70–79 were 20, 17, 18, 22, 39, 17, and 1, respectively. Of 67 placebo recipients, 47 were male, and the mean age was 49 years. The numbers of placebo subjects in age groups 18–29, 30–39, 40–49, 50–59, 60–69, 70–79, and ≥80 were 10, 10, 10, 10, 10, 17, 10, and 0, respectively.

GIA. The assay was a modification of the methods used by Sadziene et al. [25] and Ma and Coughlin [26]. Rabbit serum, gelatin, and phosphorhyalin were added to incomplete BSK II medium at the time of use [25]. A frozen aliquot of spirochetes was thawed, grown at 34°C to a density of 108 cells/mL, and diluted in medium to 4 × 107 cells/mL. Serum samples for the assay were thawed, heated at 56°C for 30 min, diluted 1 : 4 in BSK II medium, and then passed through a 13-mm-diameter polyethersulfone membrane with 0.2-μm pores (Gelman Sciences, Ann Arbor, MI). Serial 2-fold dilutions in medium were made in sterile, flat-bottom, 96-well polystyrene microtiter plates (Corning, Cambridge, MA). The outer wells around the plate were left empty. The final volume of diluted serum in each well was 50 μL, and the lowest dilution was 1 : 8. To the diluted serum were added 50 μL of the spirochete suspension and then 100 μL of BSK II containing 20 hemolytic UI/mL guinea pig complement (Sigma Chemicals, St. Louis) and an additional 120 μg/mL phenol red (Sigma). The outer wells of each plate were then filled with sterile distilled water. The plates were sealed with adhesive plate sealers (Accumed, Westlake, OH) and Parafilm (American National Can, Chicago) and were then incubated at 34°C in 2% CO2 for 66–68 h.

The assay was performed in duplicate and in a blinded manner. Each assay included rabbit polyclonal anti-OspA hyperimmune serum (provided by R. Huebner, Pasteur Merieux Connaught) and pooled nonimmune human serum (Irvine Scientific, Irvine, CA). Dual readings at 562/630 nm were taken with a spectrophotometric plate reader with SoftMax Pro software (Molecular Devices, Sunnyvale, CA) [26]. A decrease in optical density of ≥0.5 at 562/630 nm corresponded to a visual change in the medium from red to yellow. For phase-contrast microscopy, 10 μL of well contents was examined at ×400 magnification. Spirochete growth was considered inhibited if ≥90% of the cells were nonmotile, had surface blebs, or were aggregated [17]; the highest serum dilution with growth inhibition was recorded. Of the 1100 serum samples that were subjected to GIA, 870 (79%) had the same result regardless of whether GIA titers were measured by color change or by microscopy of the well contents. When there was a discrepancy between titers by each criterion, the microscopy criterion was used for titer determination.

OspA ELISA. The ELISA to measure the binding of serum IgG immunoglobulin to OspA was performed as described elsewhere [27]. Purified lipidated strain B31 OspA [6] was used at a concentration of 1.0 μg/mL, and the microtiter plates were coated with 0.1 mL of the strain per well. The highest dilution with an absorbance ≥3 SD higher than the mean for negative control wells in the same plate was the end point. The lowest dilution of serum for the OspA ELISA was 1 : 25, and the highest was 1 : 12,800.

Paradoxically high GIA titers. When the GIA titer of a sample was high and the OspA ELISA titer was low, 2 other assays were performed. The first was a GIA in which B. hermsii replaced B. burgdorferi; inhibition of both B. hermsii and B. burgdorferi indicated the presence of antibiotics or other inhibitory substance in the serum. The second assay was a whole-cell ELISA performed with B. burgdorferi B31 (OspA+) or B314 (OspA−). The plates were produced at MarDx Diagnostics (Carlsbad, CA) by the same procedures used for production of the manufacturer’s commercial whole-cell B. burgdorferi ELISA. The assay was performed according to the manufacturer’s instructions; samples with an absorbance value ≥1.2 were considered positive. Serum specimens that were positive with B314 and with B31 cells in the whole-cell ELISA were considered to contain antibodies to B. burgdorferi antigens other than OspA. Subjects whose preimmunization specimens showed evidence of the presence of antibiotics or past infection with B. burgdorferi were excluded from the analysis. After immunizations began, specimens that showed evidence of the presence of antibiotics were excluded.

Statistics. GIA and ELISA were compared by a log, dilution–point scale; for example, the lowest GIA titer of 1 : 8 was expressed as an adjusted log2 of 1, and a GIA titer of 1 : 16 was expressed as an adjusted log2 of 2. Similarly, for ELISAs, a titer of 1 : 25 was an adjusted log, of 1, and a titer of 1 : 50 was an adjusted log, of 2. Linear regression analysis, Pearson’s correlation coefficient, and the χ2 test were used. In the case-control part of our study, control subjects were matched to case subjects by sex
Figure 1. Growth-inhibition assay (GIA) of sera from 67 placebo and 134 outer surface protein (Osp) A vaccine recipients before first immunization (prevaccine), after the second immunization, before the third immunization, and after the third immunization. Geometric mean GIA titers (right) and adjusted mean GIA titers in log 2 scale (left) for 67 placebo and 134 OspA-vaccine recipients with GIA titers > 64 (log2 of 4) before immunization. For adjusted mean titers by log2 scale, value of 1 represents serum dilution of 8. Error bars are 95% confidence intervals.

Results

GIA titers after immunization with OspA. Figure 1 compares the mean GIA titers of sera from 134 OspA recipients and 67 placebo recipients who did not have Lyme disease during the course of the trial and who did not have elevated GIA titers before immunization. Serum was obtained before the first and third immunizations, and after the second and third immunizations. The mean GIA titers of OspA recipients were several-fold higher than the titers of placebo recipients after the second and third immunizations, but <2-fold higher just before the third immunization. In previous studies of vaccinated mice, a GIA titer ≥64 (log2 of 4) was predictive for protection against challenge with B. burgdorferi [19, 20, 28]. Of the 134 OspA recipients, 99 (74%) had serum GIA titers ≥64 (log2 of 4) after 3 doses of the vaccine, whereas none of the placebo subjects had a titer ≥64 at this or any other time. However, just before immunization 3, only 16% of the OspA recipients had titers ≥64.

GIA titer by sex and age. A difference was observed between subjects ≥50 years old and those <50 years old in the frequency of GIA titers ≥64 after 3 immunizations. Of 79 vaccinees ≥50 years old, 49 (62%) had a post–dose 3 GIA titer ≥64 (log2 of 4) versus 44 (80%) of 55 vaccinees <50 years old (χ² test, 4.12; P = .04). GIA titers of men and women of all ages among the vaccinees were then compared (figure 2). The overall age difference was accounted for mostly by the frequency of reduced GIA titers among older men. Regression analysis showed that the GIA titer declined more with age for men (P = .03) than for women (P = .29).

Comparison of GIA and OspA ELISA. We next compared antibody responses by OspA ELISA and by GIA among OspA recipients without Lyme disease. Sera from a randomly selected subset of 74 of the 134 subjects were tested by OspA ELISA. Figure 3 shows the correlation of GIA and OspA ELISA for 295 samples from the 74 subjects over the course of the study; the large number of samples with adjusted log2 titers of 1 includes preimmune samples. When preimmunization values were excluded, the best correlation between GIA and ELISA titers was for post–dose 2 sera (r = .70, P < .005) and post–dose 3 sera (r = .84; P < .005). The correlation coefficient for the pre–dose 3 sera was only .31 (P = .01).

If the cutoff points for the assays were an OspA ELISA titer ≥200 (log2 of 4) and a GIA titer ≥64 (log2 of 4), 141 sera were negative by both GIA and ELISA, and 95 sera were positive by both assays. For 41 sera, the OspA ELISA was positive, and the GIA was negative; only 6 sera were GIA positive and ELISA negative (χ² test, 8.39; P = .004). If a GIA titer ≥64 is considered an indicator of immunity to challenge, then an OspA ELISA titer ≥200 had a sensitivity of 94% and a specificity of...
77%. The negative predictive value for an ELISA titer <200 was 96%.

Sera from subjects with Lyme disease. Of the 80 subjects who had Lyme disease by the case criteria [11], 61 (76%) were placebo recipients, and 19 (24%) had received ≥1 dose(s) of OspA. GIA titers were determined for serum samples from all subjects with Lyme disease; OspA ELISA was also carried out on serum samples from case subjects who had received the OspA vaccine. Serum samples were obtained at the usual time points for the vaccine trial, as well as at the time of presentation for suspected Lyme disease and at follow-up for the illness. If the subject was known to be receiving antibiotics at the time the serum sample was obtained, the GIA result was excluded.

None of the 61 placebo recipients with Lyme disease had GIA titers >16 before or after immunization or during or after infection. Although the use of immunoblot analysis showed that these subjects had an antibody response to B. burgdorferi infection [11], they did not have detectable growth-inhibiting antibodies to the OspA-expressing B31 strain.

Of the 19 case subjects among OspA recipients, 9 received 3 doses of OspA and had serum sampled before immunizations 1 and 3 and at least once after immunization 3. Seven of the 9 subjects had Lyme disease 2–5 months after immunization 2; of the remaining 2 subjects, 1 had Lyme disease 0.5 months after immunization 3, and 1 had Lyme disease 3 months after immunization 3. Serum samples obtained 0.5–5 months before the onset of Lyme disease from 6 of the subjects were available. Only 1 of the 9 subjects had a preinfection GIA titer ≥64. The GIA titer of this subject was high (1024), but the OspA ELISA titer was low (50). The subject’s serum was negative when tested with the use of whole-cell ELISA with B31 and B314, and it did not inhibit the growth of B. hermsii (data not shown). Four other OspA recipients had only 2 doses of vaccine during the study and acquired Lyme disease 2–5 months after dose 2. Their GIA titers 1 month after the second dose were only 8, 16, 32, and 32, respectively.

Case-control study of GIA and ELISA. The findings in vaccinees who developed Lyme disease during the trial suggested that they had a poor response to the immunization before infection. We therefore examined differences in antibody responses, as measured by GIA or OspA ELISA, between vaccinated subjects who developed Lyme disease and those who did not. We compared GIA and ELISA titers for these groups at 2 time points—at the time of administration of dose 3 of vaccine and 1 month after administration of dose 3. Although we cannot rule out an effect of infection itself, the results in the placebo recipients who developed Lyme disease showed that elevation of the GIA titer of antibody to strain B31 B. burgdorferi does not occur with early infection. We analyzed subjects’ antibody responses after 3 immunizations with OspA and, in some cases, even after infection had occurred. The null hypothesis was that case subjects and control subjects showed no differences in antibody responses to OspA, as measured with the use of GIA or OspA ELISA.

For each vaccinee who developed Lyme disease, 3 age- and sex-matched control subjects were randomly selected who had been vaccinated but who did not have a diagnosis of Lyme disease. The 9 case subjects and 27 matched control subjects were compared with respect to their adjusted log2 GIA titers and ELISA titers before and after dose 3. The arithmetic means of each group’s GIA and ELISA titers, with 95% confidence intervals, were compared by use of Student’s t test and the nonparametric Mann-Whitney rank sum test (table 1). The groups were almost identical in mean age and sex distribution. Each statistical instrument showed a significant difference between case and control subjects with respect to the GIA and OspA ELISA titers after, but not at the time of, administration of dose 3.

The data were also analyzed by use of 2 × 2 matrices. For subjects with a GIA titer ≥64 (log2 of 4) and an OspA ELISA titer ≥200 (log2 of 4), no difference was found between case and control subjects before immunization 3: 8 of 9 case subjects...
and all 27 control subjects had a pre-dose 3 GIA titer <64 ($\chi^2$ test, 0.343; $P = .6$). All 9 case subjects and 23 of the 27 matched control subjects had a pre-dose 3 ELISA titer <200 ($\chi^2$ test, 0.375; $P = .5$). However, 1 month after dose 3, 1 of 9 case subjects and 18 of 27 matched control subjects had a GIA titer $\geq$64 ($\chi^2$ test, 6.279; $P = .01$), and 5 of 9 case subjects and 25 of 27 control subjects had an ELISA titer $\geq$200 ($\chi^2$ test, 4.267; $P = .04$). If absence of diagnosed Lyme disease after 2 or 3 doses of vaccine was taken as the end point for the case-control study, then the sensitivity, specificity, and predictive values for a post-dose 3 GIA titer $\geq$64 were 67%, 89%, and 95%, respectively. The sensitivity, specificity, and predictive value for a post-dose 3 ELISA titer $\geq$200 were 93%, 44%, and 83%, respectively.

Discussion

The modified GIA was reproducible, and colorimetric measurement of GIA titers usually agreed with the microscopic observations. Although we used the microscopic observations to determine titers for this study, we think the assay could be automated with spectrophotometry for larger sample numbers. For this reason, we prefer the term "growth inhibition," rather than "bactericidal," to describe the action of antibodies, even though the majority of spirochetes may be killed by antibody alone or in combination with complement.

The GIA survey showed that humans immunized with recombinant OspA produce antibodies that bind to OspA in its native conformation and that inhibit the growth of *B. burgdorferi*. However, the same GIA was not useful as a diagnostic test for new infections with *B. burgdorferi*. None of the placebo recipients who had onset of Lyme disease during the prospective study had a detectable elevation of GIA titers as the result of infection. These subjects had antibodies to *B. burgdorferi* by Western blot assay [11], but the antibodies did not appear to inhibit the growth of a laboratory-reared strain expressing OspA. These results are consistent with the findings of retrospective studies by Agger and Case [29] and Rouselle et al. [22]. The vaccine’s efficacy after a 3-dose schedule was 92% in the vaccine trial described elsewhere [11]. About three-quarters of the sample of vaccinees had GIA titers $\geq$64 after 3 immunizations with OspA. In an earlier study of mice immunized with a live, attenuated strain of *B. burgdorferi*, most mice with GIA titers $\geq$64 were protected from challenge with the infectious agent; however, those with titers <64 were not [28]. The qualitative results with humans were similar to those obtained with laboratory animals. However, the GIA responses of humans immunized with OspA were generally lower than those observed in immunized mice and rats [20, 21, 25, 28]. One possible explanation is that the GIA titer after immunizations 2 and 3 may have peaked before the serum sample was obtained. Titers of GIA or bactericidal antibodies in immunized rats, mice, and hamsters declined sooner than did antibody titers measured by ELISA [17, 28, 30]. Another possible reason for the lower GIA titers in humans was the participation of many older persons in the phase III study [11]. Mean GIA titers were lower in subjects $\geq$50 years old than in those <50 years old. Finally, it is possible that a GIA titer $\leq$32 in the blood is sufficient to protect humans from infection by tick transmission. In most studies of vaccinated animals involving GIA-type assays, challenge inocula were delivered by syringe. Lower antibody concentrations may be required to prevent transmission from an infected tick than to eliminate the spirochetes from an injected skin site [9, 10, 13].

The high correlation between GIA and OspA ELISA results for humans immunized with OspA was similar to our earlier observations for GIA and a whole-cell ELISA with immunized mice [28]. The retrospective case-control part of our study showed that a group of vaccinees who acquired Lyme disease after 2 or 3 vaccinations had lower mean titers, as determined with the use of OspA ELISA and GIA, than did matched controls tested after the third immunization. In this analysis, the GIA was not as sensitive as the OspA ELISA for the absence of Lyme disease among vaccinees. Thus, an ELISA for antibodies to OspA may perform sufficiently well for some assessments of immune response after immunization [31]. Neverth-
less, in the same case-control part of our study, the GIA had a higher specificity and predictive value than did the OspA ELISA. Among subjects of the phase III vaccine trial, significantly more had a high ELISA paired with a low GIA result than had a high GIA paired with a low ELISA result. Schmitz et al. [30] found a similar degree of dissociation between a bactericidal assay and a whole-cell ELISA. Padilla et al. [32] concluded that a bactericidal antibody assay of sera from hamsters immunized with OspA provided a better in vitro correlate of protection than measurement of total anti-OspA antibody by ELISA.

The discordance between GIA titers and OspA ELISA titers in some of our subjects indicates the presence in these sera of antibodies to epitopes of OspA that are not associated with growth inhibition in vitro or with protective immunity in vivo. Concentrations of antibodies to the epitope for a particular monoclonal antibody, LA-2, correlated well with protection from infection in experimental animal studies [33, 34], and, in the other human trial of OspA, LA-2 antibody titers of samples drawn at corresponding times were lower in persons who had been vaccinated and then developed Lyme disease than in subjects who received the vaccine but did not develop disease [12]. However, it is also possible that epitopes other than LA-2 are the targets of protective antibodies. Thus, reliance on a single monoclonal antibody for an assay may underestimate the diversity of protective immune responses among immunized humans.

Studies of immunized human subjects and laboratory animals have shown that vaccinees with low levels of antibody to OspA by GIA are at higher risk of acquiring Lyme disease than are those with higher levels. In the present study, several vaccinees who acquired Lyme disease had another dose of OspA after their infection and still had a poorer antibody response to the immunization than did most other vaccinees, an indication that some persons are inherently poor responders to OspA immunization.

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