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Borrelia burgdorferi Strain-Specific Osp C-Mediated Immunity in Mice

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Antibodies to the outer surface proteins (Osps) A, B, and C of the spirochete *Borrelia burgdorferi* can prevent infection in animal models of Lyme borreliosis. We have previously demonstrated that immune serum from mice infected with *B. burgdorferi* N40 can also prevent challenge infection and induce disease regression in infected mice. The antigens targeted by protective and disease-modulating antibodies are presently unknown, but they do not include Osp A or Osp B. Because Osp C antibodies are present in immune mouse serum, we investigated the ability of hyperimmune serum to recombinant Osp C (N40) to protect mice against challenge infection with N40 spirochetes. In both active and passive immunization studies, Osp C (N40) antiserum failed to protect mice from challenge infection with cultured organisms. Mice actively immunized with recombinant Osp C (N40) were susceptible to tick-borne challenge infection, and nymphal ticks remained infected after feeding on Osp C-hyperimmunized mice. In contrast, similar immunization studies performed with Osp C (PKo) antiserum prevented challenge infection of mice with a clone of PKo spirochetes pathogenic for mice. Both Osp C (N40) and Osp C (PKo) antisera showed minimal in vitro borreliacidal activity, and immunofluorescence studies localized Osp C beneath the outer membrane of both N40 and PKo spirochetes. We conclude that Osp C antibody-mediated immunity is strain specific and propose that differences in Osp C surface expression by spirochetes in vivo may account for strain-specific immunity.

Lyme borreliosis is a tick-borne multisystem infection due to the spirochete *Borrelia burgdorferi* (12). Protective humoral immunity to challenge infection with *B. burgdorferi* can be induced by both active and passive immunization and has been demonstrated for a number of outer surface proteins (Osps) (17, 18, 21, 27, 29, 31). The most widely studied of the potential vaccine antigens is the 31-kDa Osp A, a dominantly expressed Osp on North American strains of *B. burgdorferi*. While Osp A antibodies can protect against challenge infection in experimental animal models (17, 18, 28), recent studies have shown that the activity of the Osp A vaccine is directed against spirochetes in the midgut of feeding ticks, prior to entry into the mammalian host. Spirochetes expressing Osp A markedly diminish in number in their migration from tick midgut to salivary gland during tick feeding, despite massive replication of spirochetes, indicating that the majority of spirochetes entering the host bear other surface antigens (15, 16, 32). Similar expansion of non-Osp A-expressing spirochetes has been demonstrated to occur in mammals after syringe inoculation with cultured organisms (25). The findings that Osp A immunity can be overcome with high-dose syringe inoculation of cultured spirochetes, or if antibodies are passively administered 24 to 48 h after tick attachment to the host, are likely explained by changes in the level of Osp expression as the spirochete adapts to its new environment.

Because of the limitations of Osp A immunity, other *B. burgdorferi* proteins have been evaluated as potential vaccine antigen candidates. One such antigen, Osp C, a 22-kDa lipoprotein, has been demonstrated to increase in prevalence among spirochetes in feeding ticks and in the mammalian host during early infection (25, 32). Antibodies to Osp C are readily found in sera of infected humans and mice and provide a useful serologic marker of early *B. burgdorferi* infection (7, 20, 26). Osp C is expressed by many isolates of *B. burgdorferi*, but the level of expression is quite variable and tends to be inversely correlated with that of Osp A and B. The utility of Osp C as a single vaccine antigen has therefore been considered limited, even though Osp C vaccination has proven to be effective in gerbils and mice immunized with the recombinant protein (27–29). In contrast to Osp A antibodies, Osp C immunity has been evaluated for a very small number of *Borrelia* strains.

We have previously shown that serum from actively infected mice (immune mouse serum) not only protects naive mice from infection but also induces arthritis regression when passively administered to infected immunocompetent or severe combined immunodeficiency mice (4, 6). The antigens targeted by protective and disease-modulating antibodies are unknown. Of the antibodies identified by immunoblotting of cultured organisms, only those binding Osp C have the potential for conferring protection against infection, as has been demonstrated for other strains of *B. burgdorferi* (18, 21, 27, 29). The present study was designed to investigate whether Osp C antibodies induced by *B. burgdorferi* N40, the strain used in our protection studies with immune mouse serum, could prevent challenge infection with this organism.

MATERIALS AND METHODS

Mice. Specific-pathogen-free, weanling (4-week-old) or 6- to 8-week-old, female C3H/HeJ or C3H/HeNCr mice, purchased from The Jackson Laboratory (Bar Harbor, Maine) or from the Frederick Cancer Research Center (Frederick,

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Md.), were housed in filter cages and administered food and water ad libitum. Mice were sacrificed by carbon dioxide asphyxiation.

B. burgdorferi. Two cloned strains of *B. burgdorferi* were used. *B. burgdorferi* N40 was cloned by limiting dilution and mouse passaged as described previously (5). *B. burgdorferi* PKo was likewise cloned, mouse passaged, and proven to be consistently infectious and pathogenic (data not shown). The median intradermal infectious dose for both N40 and PKo was 10 cloned spirochetes (data not shown). Frozen aliquots of low-passage-cloned spirochetes were thawed and grown at 33°C in modified Barbour-Stoenner-Kelly (BSK II) medium (2). Spirochetes were assessed for viability and enumerated by dark-field microscopy using a Petroff-Hausser chamber immediately prior to use.

Generation of recombinant proteins. Several forms of recombinant rOsp C (rOsp C) were used in these experiments. All were initially generated by PCR amplification of a truncated DNA product, using N40 or PKo genomic DNA as the template. The truncated DNA eliminated amino-terminal nucleotides encoding the leader peptidase signal sequence, thereby facilitating the production of a soluble recombinant fusion protein. The PCR oligonucleotide primers used were 5′-CGCGGATCCAATAATTCAGGG-3′ and 5′-CGCGAATTCTTAAG GTTTTTTTGG-3′, complementary to the amino- and carboxy-terminus regions, respectively, of the open reading frame of PKo (20). The gene sequence for Osp C (N40) is identical to that for Osp C (PKo) in these regions (33). The amplified products were first cloned into Bluescript IIKS for sequence confirmation and then subcloned into the expression vector pGEX-2T (Pharmacia, Piscataway, N.J.), which produces the desired recombinant product linked to glutathione *S*-transferase (GT) via a thrombin cleavage site. Recombinant plasmids were transformed into *Escherichia coli* DH5α, and GT-Osp C expression was induced over 2 h by the addition of 0.5 mM isopropyl-1-thio-β-D-galactopyranoside. GT-Osp C was purified by affinity chromatography using a glutathione-agarose column (Pharmacia). GT was expressed and purified in the same way as the GT fusion protein, using the unmodified pGEX-2T expression vector for transformation. In some experiments, rOsp C was cleaved from the bound GT fusion partner with thrombin as specified by the manufacturer (Pharmacia). Thrombin was removed from the rOsp C eluates by affinity chromatography using antithrombin III-coated agarose beads (Sigma, St. Louis, Mo.). GT-Osp C (N40), rOsp C (N40), and rOsp C (PKo) each appeared as a single band by Coomassie brilliant blue staining of sodium dodecyl sulfate-polyacrylamide gels and reacted by immunoblotting with sera from mice infected with the homologous strain of *B. burgdorferi* (data not shown). Osp A was also produced in recombinant form with and without the GT fusion partner, as previously described (10).

Generation of immune mouse sera and hyperimmune sera to recombinant proteins. Four-week-old C3H mice were inoculated intradermally with 10^2 N40 and sacrificed 1 to 3 months later. Sera from mice sacrificed at identical time points were pooled once infection was verified in mice by culture of blood or internal organs. Thirty- and 90-day immune sera were derived from mice sacrificed at 30 and 90 days, respectively, after infection. Sera were stored at −70°C prior to use in the indicated experiments.

To produce hyperimmune sera to rOsp A or rOsp C, 6- to 8-week-old mice were injected subcutaneously with 20 to 50 μg of recombinant proteins emulsified in complete Freund's adjuvant (CFA). Two and four weeks after the primary immunization, mice were boosted with 10 to 20 μg of the identical antigen in incomplete Freund's adjuvant. Blood was obtained by retro-orbital bleeding 2 weeks after the final immunization, and serum titers were determined by immunoblotting using GT or the strain-specific rOsp A or C. All sera reacted with the specific immunizing Osp as well as the native protein at serum dilutions of at least 1:10,000, as determined by immunoblotting of rOsp and *B. burgdorferi* lysate (1 μg of lysate protein/lane). Rat anti-Osp C antisera (*B. burgdorferi* N40 and PKo) and control hyperimmune sera used in immunofluorescence experiments (rat antiflagellin [*B. burgdorferi* B31] and rat anti-Osp A [*B. burgdorferi* N40] antisera) were generated as described previously (14, 22). Rat anti-Osp C antiserum was produced by immunization with rOsp C (N40 or PKo) generated from the same plasmids used to produce rOsp C for mouse immunization experiments.

Generation of *B. burgdorferi*-infected nymphal ticks. Laboratory-reared *Ixodes scapularis* larvae were placed on C3H mice infected 1 month earlier with *B. burgdorferi* by syringe inoculation with 10^4 *B. burgdorferi* N40. On average, 100 larvae were placed on a single mouse. Larvae were allowed to feed to repletion and detach naturally into a water bath beneath the animal cage. Fed larvae were retrieved, and 5% of larvae were checked for spirochetes by immunofluorescence analysis. Approximately 70% of the larvae examined were infected. Larvae were stored in a humidified chamber at ambient temperature until they molted into nymphs and then subsequently used in transmission studies.

Challenge infection of mice. Mice vaccinated by active immunization were challenged 2 weeks after the last boost by intradermal inoculation into the shoulder (contralateral to the immunization site) with the indicated number of spirochetes. In some experiments, naive 4-week-old mice were passively immunized by subcutaneous injection of 0.5 ml of 1:10 dilution of hyperimmune serum to recombinant proteins obtained from mice immunized according to the protocol described above. Eighteen hours after passive immunization, mice were challenged with the indicated number of cultured spirochetes. For tick challenge, mice were first actively immunized with GT or GT-Osp C (N40) and bled, and sera were tested to verify that antibody titers were greater than 1:40,000. Immunized mice were then anesthesized with methoxyfluorane, and four *B. burgdorferi* N40-infected *I. scapularis* nymphs were placed on each mouse and allowed to attach. Nymphs were allowed to feed to repletion and detach naturally into a water bath below the animal cages, at which time they were retrieved and examined by confocal immunofluorescence microscopy for infection (16). Mice were sacrificed 2 weeks after challenge and examined for infection by culture of blood, urinary bladder, and spleens and for disease by histopathology.

Assessment of infection status and disease. At the time of sacrifice, 2 drops of blood and 2-mm urinary bladder or spleen specimens were cultured in BSK II medium for 2 weeks at 33°C. At the end of the culture period, the medium was examined by dark-field microscopy for the presence of viable spirochetes. In addition, tissues (hearts and joints) were examined by histopathology for evidence of *B. burgdorferi*-related disease. Both hindlegs and hearts were fixed in formalin, demineralized (for specimens containing bone), embedded in paraffin, sectioned, and stained with hematoxylin and eosin (8). Knees and tibiotarsal joints and hearts were evaluated blindly and tabulated or scored for the presence and severity of inflammation as previously described (6).

Localization of Osp C. Two immunofluorescence methods were used, the first examining surface-exposed antigens of viable organisms labeled in suspension and the second detecting antigen expression after methanol fixation of spirochetes (14). In the first method, 1-ml samples of spirochete cultures grown to stationary phase were aliquoted into 1.5-ml Eppendorf centrifuge tubes, and rat antisera were added at the following concentrations: GT antiserum, 1:100; Osp C antiserum, 1:40; flagellin antiserum, 1:100; and Osp A antiserum, 1:1,000. Cultures were incubated for 1 h at 34°C, after which the spirochetes were washed twice in CMRL 1066 medium (Gibco BRL) containing 10% fetal calf serum (FCS). After resuspension of spirochetes in 500 μl of 10% FCS–CMRL, 10-μl aliquots were pipetted onto slides and allowed to dry for 2 h over desiccant. Slides were then flooded with 50 μl of 10% FCS–CMRL and placed in a humidified chamber for 30 min. After draining of residual solution, slides were flooded with 50 μl of fluorescein isothiocyanate-conjugated goat anti-rat immunoglobulin diluted 1:1,000 in phosphate-buffered saline (PBS) and incubated for 30 min in a humidified chamber. After being washed three times by submersion in PBS (1 min/wash), slides were air dried and briefly submerged in distilled water to wash off residual PBS. After the slides were allowed to air dry a second time, coverslips were mounted with 10 μl of Fluoromount-G (Electron Microscopy Sciences) and the edges were sealed with clear nail polish.

Antigen expression by spirochetes was confirmed by a second immunofluorescence technique using permeabilized spirochetes. Ten-microliter aliquots of spirochete cultures grown to stationary phase were placed on glass slides and allowed to dry for 2 h over desiccant. Organisms were permeabilized by submerging slides in 100% methanol for 10 min. After air drying, slides were flooded with 50 μl of 10% FCS–CMRL and placed in a humidified chamber for 30 min. After residual medium was drained from the slides, 50 μl of primary antiserum was added and the slides were incubated for 1 h in a humidified chamber. All primary antisera were used at identical dilutions as described above for surface antigen detection, except that Osp C antisera were used at 1:250 dilution. Slides were washed three times in PBS; then secondary antibodies added, and the slides were processed as described above.

In vitro borreliacidal assay. A modification of a previously described borreliacidal assay (23) was used. Briefly, normal mouse serum, 30-day immune serum, or GT, rOsp A (N40), or rOsp C (N40 or PKo) hyperimmune serum was heat inactivated for 1 h at 56°C, sterilized by passage through a 0.22-μm-pore-size filter, and then diluted 1:10 in BSK II medium; 100 μl of each diluted serum was aliquoted into 96-well, flat-bottom microtiter plates and then diluted serially twofold in BSK II medium to a final dilution of 1:1,280. To each well were added 10^6 spirochetes in 100 μl of BSK II medium followed by 10 μl of guinea pig complement (Gibco/BRL, Gaithersburg, Md.). The mixture was then incubated at 37°C for 2 h with periodic shaking. Control assays were performed in identical fashion, except that sera were not heat inactivated and complement was omitted from the reaction. A 10-μl aliquot was mounted onto clean glass slides, and the total number of live and dead spirochetes was determined by examining 25 random fields under dark-field microscopy at a magnification of ×400. Spirochetes were considered dead if they had extensive surface blebbing or demonstrated loss of refractivity or motility. Results were compared with those for control wells which contained spirochetes in BSK II medium alone. To confirm spirochete death, 100-μl samples from each well were inoculated into 5 ml of BSK II medium and incubated at 33°C for 5 days. The number of viable spirochetes at the end of the culture period was determined by dark-field microscopy as described above.

RESULTS

Osp C (N40) immunity does not provide protection against *B. burgdorferi* N40. We have previously demonstrated that immune sera derived from mice infected with as few as 10^1 *B. burgdorferi* N40 spirochetes can prevent infection of passively immunized mice challenged with 10^4 spirochetes (4). Because these sera contain Osp C antibodies among their repertoire, we questioned whether Osp C (N40) antibodies could account for the protective capacity of the sera.

TABLE 1. Osp C (N40) hyperimmune serum does not prevent challenge infection with 10^3 *B. burgdorferi* N40 spirochetes

Treatment	Culture[a]	Arthritis[b] (avg severity)
Normal mouse serum	4	1.0
90-day immune serum	0	0
Osp A (N40) hyperimmune serum	1	0.4[c]
Osp C (N40) hyperimmune serum	4	1.3

[a] Values represent the number of mice from which spirochetes could be cultured among four mice examined in each treatment group.

[b] Values represent the average arthritis severity score (on a scale of 0 to 3) calculated by dividing the sum of the highest tibiotarsal scores for all mice by the total number of mice examined in each group.

[c] Arthritis was present only in the mouse in this group that had culture evidence of spirochete infection.

We first examined whether mice passively immunized with Osp C (N40) hyperimmune serum were susceptible to challenge infection with *B. burgdorferi* N40 (Table 1). Consistent with previous reports, all mice passively immunized with 90-day immune serum and three of four mice administered Osp A hyperimmune serum were protected from infection. In contrast, none of the mice passively immunized with Osp C hyperimmune serum resisted challenge inoculation with 10^3 spirochetes. To ensure that the lack of protection with Osp C hyperimmune serum was not due to suboptimal antibody levels, we actively immunized mice with rOsp C (N40) prior to challenge inoculation. In the group of seven mice immunized with CFA, six had spirochete-positive cultures and six showed signs of disease (disease was considered present if hearts or joints showed any evidence of inflammation by histopathology). In the group of seven mice immunized with Osp C (NO) plus CFA, seven had spirochete-positive cultures and six showed signs of disease. Mice immunized with rOsp C (N40) or GT-Osp C (N40) were still susceptible to infection with *B. burgdorferi* N40, despite prechallenge antibody titers against rOsp C of >1:40,000 (Table 2).

One potential explanation for the inability of Osp C (N40) immunization to protect against infection is that the spirochetes used for challenge contained a mixture of Osp C-expressing and -nonexpressing organisms. Because Osp A antibodies protect naive mice in this experimental system, it is likely that the majority of pathogenic spirochetes introduced into the mice expressed Osp A. However, Osp A immunity can be overcome with challenge inocula of $>10^4$ spirochetes (18). This may be due to downregulation of Osp A and upregulation of Osp C by spirochetes after inoculation into mice (25). We therefore tested the ability of Osp C (N40) antibodies to enhance the protective capabilities of active Osp A immunity. Mice were immunized, boosted with either a single immunogen (GT, GT-Osp A, or GT-Osp C) or GT-Osp A in combination with GT-Osp C, and then challenged with a range of spirochete doses (Table 2). As previously observed, mice immunized with GT-Osp A were protected from infection with a challenge dose of 10^2 spirochetes, but this immunity was overcome with higher-dose challenge inocula (18). In contrast, mice immunized with GT-Osp C were not protected from infection, even at the lowest challenge dose of spirochetes. Mice immunized with the combination of GT-Osp A and GT-Osp C, although immune to the 10^2 inoculum, all became infected when challenged with higher numbers of spirochetes. The protective immunity provided by GT-Osp A immunization was therefore not enhanced by simultaneous immunization with GT-Osp C. Moreover, this active immunization study confirmed the results of the earlier experiment (see above) and showed that spirochete dose did not contribute to the failure of Osp C antibodies to provide immunity.

We also examined the ability of Osp C vaccination to prevent *B. burgdorferi* infection introduced through its natural vector, the tick. As discussed earlier, spirochetes present in the midgut of ticks express abundant amounts of Osp A, whereas during ingestion of the blood meal by feeding ticks, spirochetes that migrate to the salivary gland no longer express significant amounts of Osp A (16). In contrast, Osp C can be readily detected by immunofluorescence in feeding ticks (32). It is likely, therefore, that most of the spirochetes inoculated by tick bite express abundant amounts of Osp C. Of four mice actively immunized with GT-Osp C (N40), none were immune from infection by tick challenge. All four GT-Osp C (N40)-immunized mice were infected as determined by culture, and all had arthritis and carditis that were indistinguishable from those of control infected mice. In addition, 100% of nymphal ticks retrieved after feeding on GT-Osp C (N40)-immunized mice (11 recovered/16 total) were still infected, as determined by confocal immunofluorescence microscopy, so that unlike borreliacidal Osp A antibodies, Osp C (N40) antibodies did not eliminate spirochetes from the tick.

rOsp C (PKo) can induce protective immunity against *B. burgdorferi* PKo. We have previously shown that a GT-Osp A fusion fragment containing the amino acid sequence bound by protective Osp A mAb does not induce protective immunity, even though antibodies that bind this sequence upon immunoblotting can be elicited (11). It is possible that the secondary structure of the GT-Osp A fusion fragment did not permit priming of B cells specific for the epitope bound by protective antibodies. Because all forms of rOsp C (N40) used in the immunization experiments were first generated as fusion proteins with GT, it was necessary to show that rOsp C generated by the pGEX-2T expression system could elicit protective immunity against *B. burgdorferi* strains in which Osp C antibody-mediated protection has been described.

Immunization of gerbils with rOsp C from *B. burgdorferi* PKo has been reported to protect them from challenge infection with spirochetes of that strain (27). Because the PKo strain used in those studies was not infectious for mice, we cloned and mouse passaged the strain. Mice inoculated with as few as 10 mouse-adapted PKo spirochetes became infected and developed both arthritis and carditis within 14 days of infection (data not shown). To determine whether hyperimmune serum to Osp C (PKo), produced as a fusion protein with GT, could prevent challenge infection with PKo spirochetes, naive mice were passively immunized with hyperimmune GT-Osp C (PKo) serum and then challenged with 10^4 PKo spirochetes. All four mice passively immunized with GT-Osp C (PKo), and none of four mice given normal mouse serum only,

TABLE 2. Active immunization with both Osp A and Osp C does not enhance protective capabilities of Osp A antibodies against different challenge doses of *B. burgdorferi* N40 spirochetes

Immunizing antigen	Culture results of mice inoculated with the indicated dose of N40 spirochetes[a]		
	10^2	10^4	10^6
GT	3/4	4/4	4/4
GT-Osp C	4/4	4/4	4/4
GT-Osp A	0/3	4/4	4/4
GT-Osp A + GT-Osp C	0/4	4/4	4/4

[a] Values represent the number of mice with positive spirochete cultures over the total number of mice examined.

were protected from infection, indicating that Osp C expressed as a GT fusion protein could elicit protective Osp C antibodies for some strains of *B. burgdorferi*. Mice immunized with GT-Osp C (PKo) had the same Osp C antibody titers against the autologous Osp C as mice immunized with GT-Osp C (N40). These results further suggested that the inability of Osp C (N40) antibodies to prevent infection was strain specific.

Antibody localization of Osp C epitopes by immunofluorescence and in vitro borreliacidal assays. It is generally believed that in order for antibodies to kill *B. burgdorferi*, they must bind surface-exposed determinants on the organism that are present in sufficient amounts to inhibit motility and induce death. Differences in the membrane location of Osp C expressed by spirochetes could provide an explanation for the apparent strain specificity of Osp C immunity. We examined this issue by comparing immunofluorescence staining of spirochetes labeled in suspension with staining of those labeled after methanol fixation. The methods used have proven to differentiate surface-exposed determinants from those located beneath the outer membrane (14). Whereas Osp A could be detected on both N40 and PKo spirochetes labeled in suspension or after fixation, Osp C could be detected only after fixation (Fig. 1). The beaded fluorescent pattern of labeled Osp A surface-exposed antigenic determinants is characteristic for this outer membrane protein, and it is likely due to surface aggregation of Osp A due to antibody-mediated cross-linking (3, 14). Similar to the requirements for immunofluorescence staining of Osp C, fixation was necessary to label spirochetes with antibodies against flagellin, a protein localized to the periplasmic space. Because Osp C is membrane bound, we infer from these studies that the majority of this protein resides below the surface, attached to the cytoplasmic membrane.

We also used an in vitro borreliacidal assay as an alternative method to assess Osp C surface expression on the two spirochete strains (Table 3). Immune serum from mice infected with *B. burgdorferi* for 30 days killed N40 spirochetes in a dose-dependent fashion, as did Osp A (N40) hyperimmune serum. These same sera also exhibited modest killing of PKo spirochetes at high serum concentrations. In contrast, Osp C (N40) and Osp C (PKo) antisera exhibited the same degree of weak killing activity against the autologous strain, which was apparent only at high serum concentration. Taken together with the data from immunofluorescence studies, these results indicate that the majority of Osp C is not surface exposed on cultured N40 or PKo spirochetes.

DISCUSSION

Humoral immunity provides an important host defense against infection with *B. burgdorferi*, and protective antibodies that recognize the outer membrane-associated lipoproteins Osps A, B, and C have been identified. Our recent work has demonstrated the capacity of immune serum from mice infected with low numbers of N40 spirochetes to both protect mice from challenge infection and induce disease regression (4, 6). In the present studies, we examined whether Osp C antibodies contributed to the protective effects of immune mouse serum because Osp C is one of the few antigens recognized by this serum on immunoblots of cultured organisms. We were surprised to find that, in contrast to reports using other strains of spirochetes (21, 27, 29) and studies suggesting that Osp C is upregulated in the feeding tick (32), antibodies to Osp C (N40) did not protect mice from syringe or tick-borne challenge and did not augment Osp A-mediated immunity.

We have previously reported that some forms of recombinant fusion proteins produced by using the pGEX bacterial expression system do not elicit protective immunity, presumably because the fusion protein cannot assume a secondary or tertiary structure required to prime B cells specific for the protective epitope (11). Although the epitopes on Osp C recognized by borreliacidal antibodies are unknown, Osp C (N40) and Osp C (PKo) are similar in size, and the GT fusion partner did not interfere with the ability of GT-PKo to elicit protective immunity. We infer from this result that altered conformation of the recombinant protein was less likely to play a role in the inability of Osp C (N40) to elicit protective immunity.

The level of surface expression of a *B. burgdorferi* protein may be a strong determinant of antibody-mediated borreliacidal activity. For example, Osp C but not Osp A antibodies provide immunity against infection with PKo spirochetes, a European isolate of *B. burgdorferi* that expresses a large amount of Osp C and relatively little Osp A (27). In contrast, antibodies to either Osp A or Osp C can protect mice against infection with SON 188, a California *B. burgdorferi* sensu stricto strain that expresses both Osps (29). In that study, in vitro borreliacidal activity could be demonstrated with Osp A antiserum, not with Osp C antiserum. It was postulated that these antisera may differ in the ability to activate complement and that Osp C antibodies may mediate spirochete killing indirectly through opsonization and subsequent enhancement of phagocytosis in vivo. However, complement is not required for in vitro borreliacidal activity, and Osp A antibodies can protect C5-deficient mice from challenge infection (9). Neither of the studies showing Osp C-mediated immunity examined the relative surface expression of Osps A and C on spirochetes used for challenge infection.

In the present study, surface immunofluorescence labeled Osp A on both N40 and PKo spirochetes, and Osp A antiserum killed both strains of spirochetes efficiently in vitro and, in the case of N40 spirochetes, also in vivo. In contrast, Osp C could not be detected on the surface of either strain of spirochete, and in vitro borreliacidal activity was apparent only at the highest antiserum concentrations. Although we did not examine the level of Osp A and Osp C expression on the original PKo strain prior to mouse passaging and cloning, it is possible that these procedures selected for spirochetes that preferentially express Osp A on the outer membrane in culture. The polyclonal Osp antisera generated for these experiments contained high titers of antibodies to the immunizing antigens and, as shown in the immunofluorescence studies, could clearly bind to the native proteins. However, it has been demonstrated for Osp A that only a subset of antibodies induced by immunization or arising naturally after infection are borreliacidal. We were able to protect mice from *B. burgdorferi* infection by active immunization with GT-Osp A or by passive administration of Osp A hyperimmune serum, with similar thresholds at which immunity could be overcome ($>10^3$ spirochetes). The relatively low titer of Osp A hyperimmune serum at which borreliacidal activity could be detected in vitro (albeit using 10^6 spirochetes) suggests that our in vitro borreliacidal assay is a less sensitive method than animal challenge experiments for assessing protective capabilities of *Borrelia* antisera. Indeed, despite the similarities between Osp C expression and Osp C antibody-mediated killing of the two *B. burgdorferi* strains in vitro, only Osp C (PKo) antibodies could protect mice from challenge inoculation against the homologous *Borrelia* isolate. These data underscore the need to be cautious in assuming that in vitro borreliacidal activity can be extrapolated to in vivo protective capacity, especially when the former is observed only at high antiserum concentrations.

An attractive explanation for the differences between the in vitro and in vivo borreliacidal activity of the hyperimmune sera

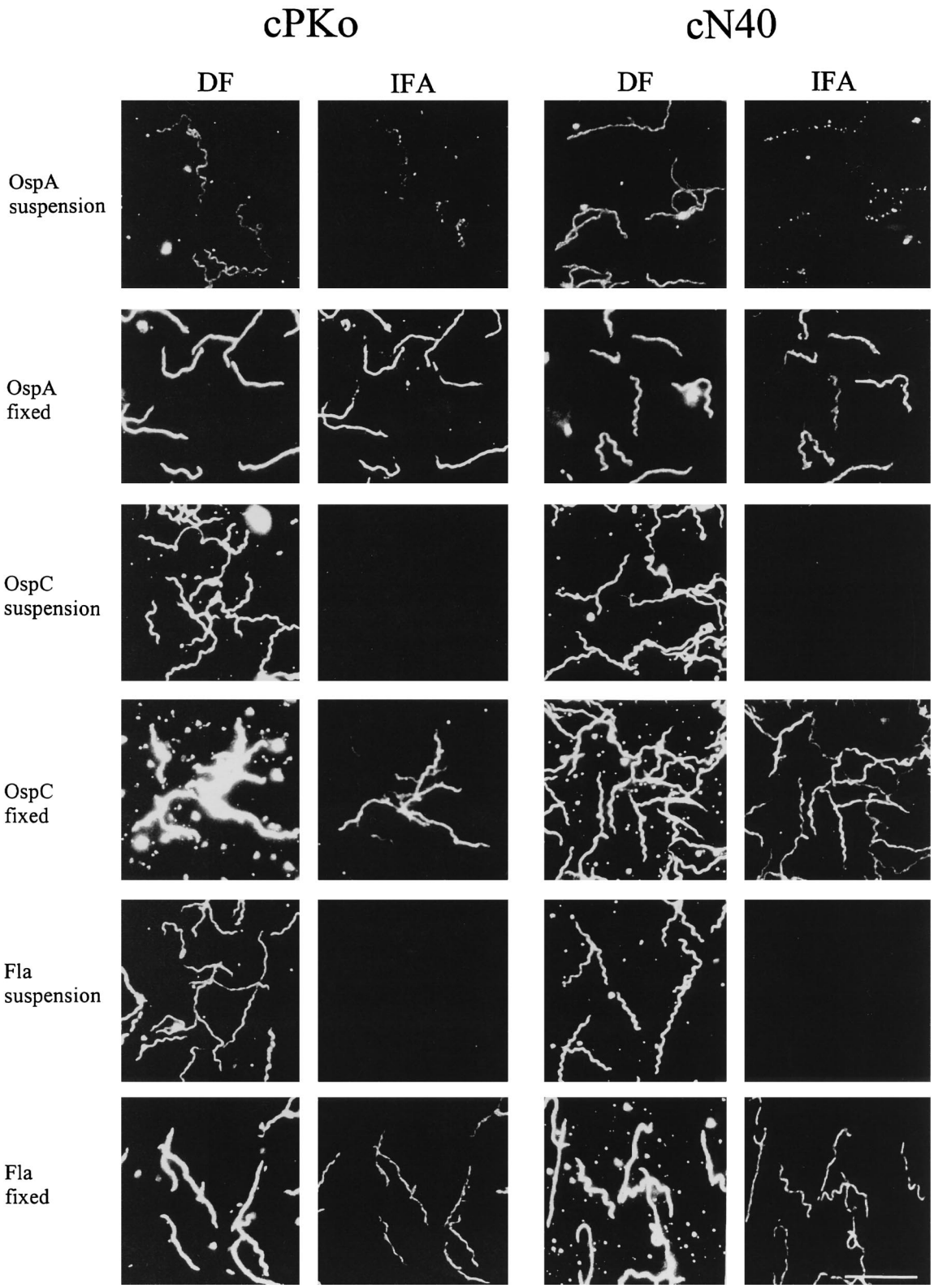

FIG. 1. Immunofluorescence analysis of Osp A, Osp C, and flagellin expression on cloned N40 and PKo spirochetes. Spirochetes were grown to stationary phase and then labeled in suspension or after methanol fixation as described in Materials and Methods. Osp C was labeled with Osp C hyperimmune sera generated from the autologous strain. Osp A was labeled with hyperimmune serum from *B. burgdorferi* N40 and flagellin (Fla) with hyperimmune serum from *B. burgdorferi* B31. DF, dark-field microscopy; IFA, immunofluorescence analysis.

TABLE 3. In vitro borreliacidal activity of Osp C (N40) and Osp C (PKo) hyperimmune serum

Treatment	LD_{50}[a]	
	N40 spirochetes	PKo spirochetes
Normal mouse serum	<1:10	<1:10
30-day immune serum	1:80	1:10
GT hyperimmune serum	<1:10	<1:10
Osp A (N40) hyperimmune serum	1:80	1:10
Osp C (N40) hyperimmune serum	1:10	<1:10
Osp C (PKo) hyperimmune serum	1:10	1:10

[a] LD_{50}, level at which >50% of 10^6 target spirochetes were killed, as initially assessed by dark-field examination and then verified by the absence of spirochete growth after a 5-day culture of 50% of the assay sample in BSK II medium.

is that the relative level of surface expression of Osp epitopes targeted by protective antibodies changes on spirochetes after inoculation into mice. California *B. burgdorferi* isolates such as SON 188 are more variable in lipoprotein expression than other North American strains (29, 35). In this regard, they appear phenotypically more like European *Borrelia* species, such as *B. burgdorferi* PKo. As noted above, Osp C antibodies to SON 188 exhibit no in vitro borreliacidal activity yet, like Osp C (PKo) antibodies, are capable of providing protection against challenge infection. It is possible that upon entry into the mammal, PKo and SON 188 spirochetes upregulate Osp C surface expression to a level sufficient for borreliacidal antibodies to bind, whereas N40 spirochetes do not. Immunofluorescence analysis of Osp C expression on spirochetes examined directly ex vivo is best performed with fixed and permeabilized organisms (25), and so this method would not distinguish between surface and subsurface location of the protein. Osp C antibodies specific for another North American isolate of *B. burgdorferi* sensu stricto, 297, a strain more closely related genetically to N40 than PKo or SON 188, also show no surface labeling and do not protect against challenge infection (30). Protection experiments using Osp C from a larger number of European and North American *Borrelia* isolates would be required to obtain statistical verification for the trend noted here.

Our results have important implications for survival strategies of *B. burgdorferi* in the mammalian host. The lipoproteins of *B. burgdorferi* are proinflammatory (24), and their surface exposure on intact spirochetes likely serves as an important signal to innate immune cells that a pathogen is present. When levels of surface expression fall below a critical threshold, the recognition of non-self may be defeated. Such a strategy may explain the apparent downregulation of Osp A on spirochetes prior to and upon entry into the mammal and the ability of spirochetes to persist in the extracellular matrix of chronically infected animals without an associated inflammatory response.

We began these studies to determine the identity of the antigens targeted by *B. burgdorferi* protective and disease-modulating antibodies present in immune mouse serum. Osp C antibodies do not appear to account for this activity. Osp C antisera also showed no arthritis-modulating effects in actively infected mice (6). It is possible that the effector antibodies in immune sera may be directed toward proteins of the spirochete expressed solely after mammalian infection. A number of *B. burgdorferi* proteins have recently been identified that appear to be preferentially expressed in vivo (1, 13, 19, 34). Further studies are currently in progress to investigate the humoral repertoire and the spirochetal antigens targeted early in the course of *B. burgdorferi* infection.

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