Direct Demonstration of Antigenic Substitution of *Borrelia burgdorferi* Ex Vivo: Exploration of the Paradox of the Early Immune Response to Outer Surface Proteins A and C in Lyme Disease

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Summary

The outer surface proteins (Osps) of *Borrelia burgdorferi*, the etiologic agent of Lyme disease, are principle targets of protective immune responses against this organism. Whereas most North American strains of *B. burgdorferi* in culture express an abundant amount of Osp A, antibodies to this protein are either absent or only weakly detected in the sera of naturally infected patients or experimentally infected mice. In contrast, Osp C, which has variable expression on cultured organisms, elicits an early, strong humoral response. To examine this paradox, we have studied the in vivo adaptation of a cloned population of *B. burgdorferi* strain N40 during the early course of experimental murine borreliosis. As in human disease, antibodies to Osp A were only weakly present in the early immune repertoire after murine inoculation with low dose (10^3) spirochetes. In contrast, antibodies to Osp C were prominent, even though on cultured spirochetes Osp C mRNA and protein expression could not be detected by reverse transcription polymerase chain reaction (RT-PCR) or indirect immunofluorescence, respectively. These observations led us to investigate the expression of Osp A and Osp C in vivo. By direct fluorescent staining of uncultured spirochetes ex vivo and by PCR amplification of spirochetal mRNA, we show that Osp C is indeed expressed by some spirochetes after infection in the mouse. Spirochetes expressing Osp A could also be detected within the first 2 wk of infection, but not at 30 d. Osp A mRNA, although present at day 14 of infection, could not be amplified by RT-PCR at day 30, suggesting that the expression of this Osp is transient. This further implies that the late burst in Osp A antibodies in both mice and humans may be anamnestic. These results indicate that either Osp C is upregulated on spirochetes after infection, or Osp C–expressing spirochetes expand preferentially over those expressing Osp A during infection. These results have important implications for vaccine design and offer one explanation for the failure of Osp A antibodies to eradicate spirochetes from the infected host.

Lyme disease, caused by infection with the tick-transmitted spirochete *Borrelia burgdorferi*, is manifested by disease that reflects the in vivo migration of this organism in the mammalian host. After establishing infection in the skin at the site of the tick bite, a transient rash, erythema chronicum migrans, can appear and is the clinical hallmark of Lyme disease. Spirochetes subsequently become blood borne and result in disease manifestations of other organs, especially the heart, nervous system, and joints (1). Although most patients are readily cured by antibiotic therapy (1), rare patients have persistent or recurring Lyme disease, which can be severe and debilitating. From the first description of the human disease, arthritis was observed to come and go, even though the patient remained infected (2). Although spirochetes become more difficult to find as the disease progresses, there is reason to believe that they are driving the illness throughout its course. Evidence in favor of a reservoir of live spirochetes in humans includes the expanding antigen specificity of immune sera from patients in later stages of disease, suggesting newly exposed spirochetal epitopes (3), and the occasional identification of spirochetes in organs clinically asymptomatic (4) or affected in late stages of disease (5, 6). In chronically infected mice, despite resolution of their disease, spirochetes are still occasionally sighted and can be cultured from various organs. And despite the apparent dormancy of the spirochetes in their original murine host, they remain infective for naive mice (7).

Outer surface protein (Osp)[1] A is a major outer mem-

[1] *Abbreviations used in this paper:* BSK II medium, Barbour–Stoener–Kelly II medium; NMS, normal mouse serum; cN40, *Borrelia burgdorferi* strain N40; Osp, outer surface protein; RT, reverse transcription.

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brane protein expressed on cultured *B. burgdorferi*, sensu stricto, the major genotype found in the United States (8). Humoral immunity to Osp A has been shown to be important in protection against infection with *B. burgdorferi* (9–12), and this antigen is now the basis of recombinant Lyme disease vaccines. Osp A vaccination, however, even 24 h after introduction of the spirochete into the host, does not eliminate infection (13). Furthermore, naturally arising Osp A antibodies are incapable of completely eradicating spirochetes from the infected host, but when passively supplied, some sera containing Osp A antibodies can protect naive mice from challenge infection (14). These findings suggest that Osp A expression on spirochetes may change during mammalian infection. The inability to visualize organisms by immunofluorescence in infected tissues and the paucity of organisms in general has made analysis of this question difficult. Organisms recultured from infected animals have identical protein and genetic make-up to the original infecting spirochetes (15); this observation may be due to in vitro growth conditions favoring reversion to the phenotype of the original culture.

Patients with Lyme disease rarely have detectable serum antibodies to Osp A during the first few months of untreated infection. A striking finding has been the consistent, strong early antibody response to Osp C, a 22-kD lipoprotein, which, in contrast to Osp A, has variable expression on cultured *B. burgdorferi* (16–20). Over time, a small proportion of patients, generally those with chronic arthritis, seroconvert to Osp A (21). Mice experimentally infected by tick challenge or syringe inoculation with low numbers of spirochetes ($<10^4$) readily develop Osp C antibodies but do not have detectable IgG antibodies to Osp A until 6 mo or later in the course of infection (22, 23). Several hypotheses have been proposed to explain the Osp A antibody response: (*a*) Osp A antibodies may be sequestered in immune complexes (24); (*b*) sufficient T cell help to promote B cell antibody production is not present (Bockenstedt, L. K., E. Fikrig, S. W. Barthold, R. A. Flavell, and F. S. Kantor, manuscript submitted for publication); and (*c*) Osp A may be downregulated on spirochetes either in the tick or after transmission to the host (25a,b). Although evidence is available to support all of these hypotheses, little is known about the proteins expressed on *B. burgdorferi* in vivo in the mammalian host. The present studies using a cloned population of *B. burgdorferi* were designed to investigate the paradox of the Osp A and Osp C humoral response.

Using a well-characterized cloned population of spirochetes, we investigated Osp A and Osp C expression early in the course of *B. burgdorferi* infection in mice. Infecting organisms expressed high levels of Osp A on their surfaces, whereas Osp C expression could not be detected at the mRNA or protein level. In contrast to the cultured organisms, some spirochetes obtained from the peritoneal cavities of infected mice and examined directly expressed Osp C but not Osp A. Osp A^+/Osp C^- spirochetes were still visualized at day 14, but were not detected at day 30. The immunofluorescent data were supported by mRNA analysis of Osp A and Osp C expression at infection days 14 and 30. These studies indicate that after infection, Osp C expression is either upregulated, or a minor population of Osp C–expressing spirochetes preferentially expands in the infected host. In contrast, Osp A–expressing spirochetes appear to diminish in number so that by day 30, they are below the level of detection. These findings may help to explain the antibody responses to these Osps in the early phases of infection, and they offer one explanation for the failure of Osp A antibodies to effectively clear spirochetes from the infected host.

Materials and Methods

Mice. Pathogen-free weanling female C3H/HeJ ($H2^k$) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Mice were shipped in filter-equipped crates and housed in microisolator cages. Animals were provided food and water ad libitum and were cared for according to the Public Health Service guide for the care and use of laboratory animals. Mice were killed with carbon dioxide gas.

Spirochetes. A low in vitro passage of *B. burgdorferi* strain N40 (cN40), which had been previously cloned and tested for infectivity and pathogenicity (22), was used in all experiments. The spirochetes had been cloned by limiting dilution culture in modified Barbour–Stoenner–Kelly II (BSK II) medium three times and then stored at −70°C. Before each experiment, an aliquot of cN40 was grown to logarithmic phase in modified BSK II medium and counted in a hemocytometer under dark-field microscopy.

Immunofluorescent Staining. Spirochetes from lavage fluids were labeled either after adherence to peritoneal macrophages on coverslips or on resuspension after pelleting. Macrophages were plated as described in αMEM/10% FBS on 12-mm glass coverslips at 37°C and fixed 15–30 min later to minimize phagocytosis (26). Spirochetes to be labeled in suspension were first pelleted in an Eppendorf microcentrifuge for 2 min at room temperature and then resuspended in PBS (Ca^{2+} and Mg^{2+} free) and fixed in methanol (−20°C, 6 min) or 4% paraformaldehyde in PBS ±0.01% saponin (room temperature, 20 min) as described (27). All samples were washed in PBS and then incubated for 20 min in PBS supplemented with 10% goat serum. Primary antibody at a dilution of 1:100 was added in PBS/10% goat serum for 1 h at room temperature, samples were washed two times in PBS, and then incubated again for 20 min in PBS/10% goat serum at room temperature. Antibodies were visualized with $F(ab)'_2$ fluorescein (FITC)- or rhodamine (TRITC)-conjugated goat anti–rabbit or anti–mouse secondary antibodies (Tago, Inc., Burlingame, CA) diluted in PBS/10% goat serum and incubated with the spirochetes for 1 h at room temperature. Samples were mounted with moviol (Calbiochem Corp., La Jolla, CA) and examined on a microscope (Axioskop; Carl Zeiss, Inc., Thornwood, NY).

Detection of mRNA. mRNA from cultured cN40 was prepared in the presence of J774 cells, a macrophage-like cell line, to simulate the in vivo conditions from which mRNA would be derived from infected tissues. 10^4 cN40 from culture were washed twice in PBS, mixed with 3×10^6 J774 cells, centrifuged, and the pellet was resuspended in 6 ml GIT buffer (4 M guanidine isothiocyanate, 0.025 M sodium acetate, pH 6.0, and 0.8% 2-ME). The DNA was first sheared by passing the solution through a 23-gauge needle 10 times, and then layered onto 4 ml of cesium chloride. The cesium chloride gradient was centrifuged at 32,000 rpm in an SW41 rotor (Beckman Inst., Inc., Fullerton, CA) for 18 h at room temperature. The RNA pellet was then solubilized in 300 μl 0.3 M sodium acetate, pH 6, precipitated with ethanol, washed once in 80% ethanol, and allowed to air dry. Complementary

DNA was transcribed from RNA (10 μg/reaction, 50 μl total reaction volume) with random hexamers using the Superscript Preamplification System for First Strand cDNA Synthesis according to the manufacturer's protocol (GIBCO BRL, Gaithersburg, MD). One fifth of the reaction volume was amplified by PCR using oligonucleotide primers for flagellin, Osp A, and Osp C at a final concentration of 10 μM each in the presence of 20 mM dNTPs. PCR was carried out for 25 cycles, with each cycle consisting of initial template denaturation at 94°C for 1 min, annealing at 50°C for 2 min, and extension at 72°C for 3 min. Amplication was completed by a final incubation at 72°C for 10 min. 10 μl out of a 100-μl reaction volume were analyzed by agarose gel electrophoresis for the predicted product. As controls for DNA contamination, identical first-strand synthesis reactions were performed in the absence of reverse transcriptase (RT) (Superscript II, RT, GIBCO BRL). mRNA from infected tissues was obtained both by the method described above (4 ml GIT/heart or lavage sample) as well as by using the Tri-Reagent RNA isolation kit (Molecular Research Center, Inc., Cincinnati, OH) following the manufacturer's recommendations. RT-PCR of infected tissue specimens was performed identically to that described above for cultured organisms, except that actin was amplified instead of flagellin. The sequences of primers and predicted amplified product sizes are as follows: 5′ flagellin AGAGAATTCAGGAGAATTTATGATTATCAATCATAATAC; 3′ flagellin TGTTTTGTCATTGCTTAGATAAGGATCCTCT (1,038 bp); 5′ Osp A CCGGGATCCATATGGCTAAGCAAAATGTTAGC; 3′ Osp A AACGCTTTAAAATAAAGATCTAGATATC (801 bp); 5′ Osp C CGCGGATCCAATAATTCAGGG; 3′ Osp C CCAAAAAAACCTTAAGAATTCGCG (590 bp); and 5′ actin GTGGGCCGCTCTAGGCACCA; 3′ actin CCCCCCTGAACCCTAAGGCCAACC (245 bp). All primers were either made at the Oligonucleotide Synthesis laboratories at Yale University or were obtained commercially (actin primers; Stratagene Inc., La Jolla, CA).

Immunoblotting. Recombinant Osp A strain N40 prepared according to the method of Dunn et al. (28) was used. Recombinant Osp C, N40, was generated first as a fusion protein with glutathione transferase using the pGEX-2T expression vector (Pharmacia Fine Chemicals, Piscataway, NJ) and then cleaved from the fusion partner using thrombin as described (20). Spirochetal lysates were prepared by washing bacteria twice in PBS, freeze-thawing once, then sonicating 10 times with 15-s pulses at 60% power. The recombinant proteins or cN40 lysates were heated to 100°C for 5 min in SDS sample buffer (50 mM Tris/Cl, pH 6.8, 100 mM dithiothreitol, 2% SDS, 0.1% bromophenol blue, 10% glycerol) before separation by SDS-PAGE. Electrophoresed proteins were then transferred to nitrocellulose using a minigel system (Bio Rad Laboratories, Hercules, CA). The strips were blocked with 5% nonfat dry milk in PBS, and primary antibodies were added at a 1:1,000 dilution of normal or immune mouse serum or 1:1,000 dilution of hyperimmune polyclonal antisera to rOsp A or rOsp C. The nitrocellulose strips were incubated for 2 h with primary antibodies and then washed three times in PBS + 0.1% Tween 20 (PBST). The strips were incubated for 2 h with a 1:5,200 dilution of alkaline phosphatase–labeled goat anti-mouse IgM or IgG antibodies (Hyclone Laboratories, Inc., Logan UT). After three 10-min washes in PBST, the strips were developed with nitroblue tetrazolium 5-bromo 4-chloro-indolyl phosphate.

Results and Discussion

We first characterized the expression of Osps A and C on low passage cultured spirochetes (previously cloned by limiting dilution) by three methods: immunoblot of spirochetal lysates, mRNA analysis, and indirect immunofluorescence of whole spirochetes. Immunoblots of lysates of cN40 (equivalent to 2×10^5 spirochetes per lane) were performed using polyclonal antisera generated against recombinant Osp A or Osp C (Fig. 1). Whereas Osp A was readily visualized (Fig. 1, lane *3*), Osp C expression could not be detected (Fig. 1, lane *5*), even though both antisera readily bound their rOsp, respectively (rOsp A, lane *2*; rOsp C, lane *4*). To simulate the conditions under which *B. burgdorferi* mRNA would be detected in infected tissues, mRNA was derived from 10^4 cN40 mixed with 3×10^6 J774 cells, a macrophage cell line, as a source for eukaryotic RNA. RT-PCR performed on 10 μg of RNA easily amplified both flagellin and Osp A mRNA (Fig. 2, lanes *6* and *7*, respectively), but no product could be detected for Osp C (Fig. 2, lane *8*). By indirect immunofluorescence, cultured N40 spirochetes (grown in BSK II medium and labeled as described [28]) were brightly stained with antibod-

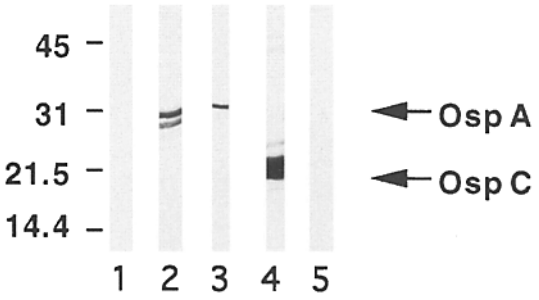

Figure 1. Osp A but not Osp C can be detected by immunoblot of cN40 lysates with polyclonal antisera to rOsps A and C. (Lane *1*) Immunoblot of rOsp C with normal mouse serum (1:100). (Lanes *2* and *3*) Immunoblots of rOsp A and cN40 lysate, respectively, with polyclonal anti-Osp A antisera (1:1,000). (Lanes *4* and *5*) Immunoblot of rOsp C and cN40 lysate, respectively, with polyclonal anti-Osp C antisera (1:1,000). Lane *1* is representative of similar immunoblots of rOsp A and cN40 with NMS.

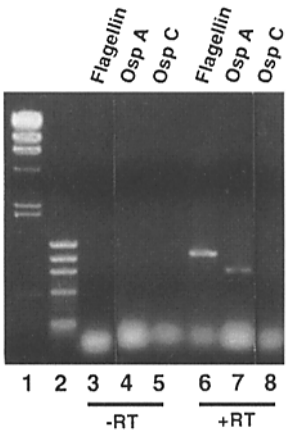

Figure 2. mRNA for flagellin and Osp A, but not for Osp C, can be detected by RT-PCR on cultured cN40. mRNA isolated from cultured spirochetes was used to produce first-strand cDNA using random hexamers as described in Materials and Methods. Flagellin, Osp A, and Osp C cDNAs were amplified by PCR and analyzed by agarose gel electrophoresis. (Lane *1*) High molecular weight marker (HindIII digest of λ bacteriophage; New England Biolabs, Inc., Beverly, MA). (Lane *2*) Low molecular weight marker (HAEIII digest of ϕX174; New England Biolabs, Inc.). (Lanes *3–5*) PCR of mock first-strand reactions using flagellin- (lane *3*), Osp A- (lane *4*), and Osp C- (lane *5*) specific primers. (Lanes *6–8*) PCR of cDNA using flagellin- (lane *6*), Osp A- (lane *7*), and Osp C- (lane *8*) specific primers. Predicted sizes of amplified products are flagellin, 1,038 bp; Osp A, 801 bp; and Osp C, 590 bp.

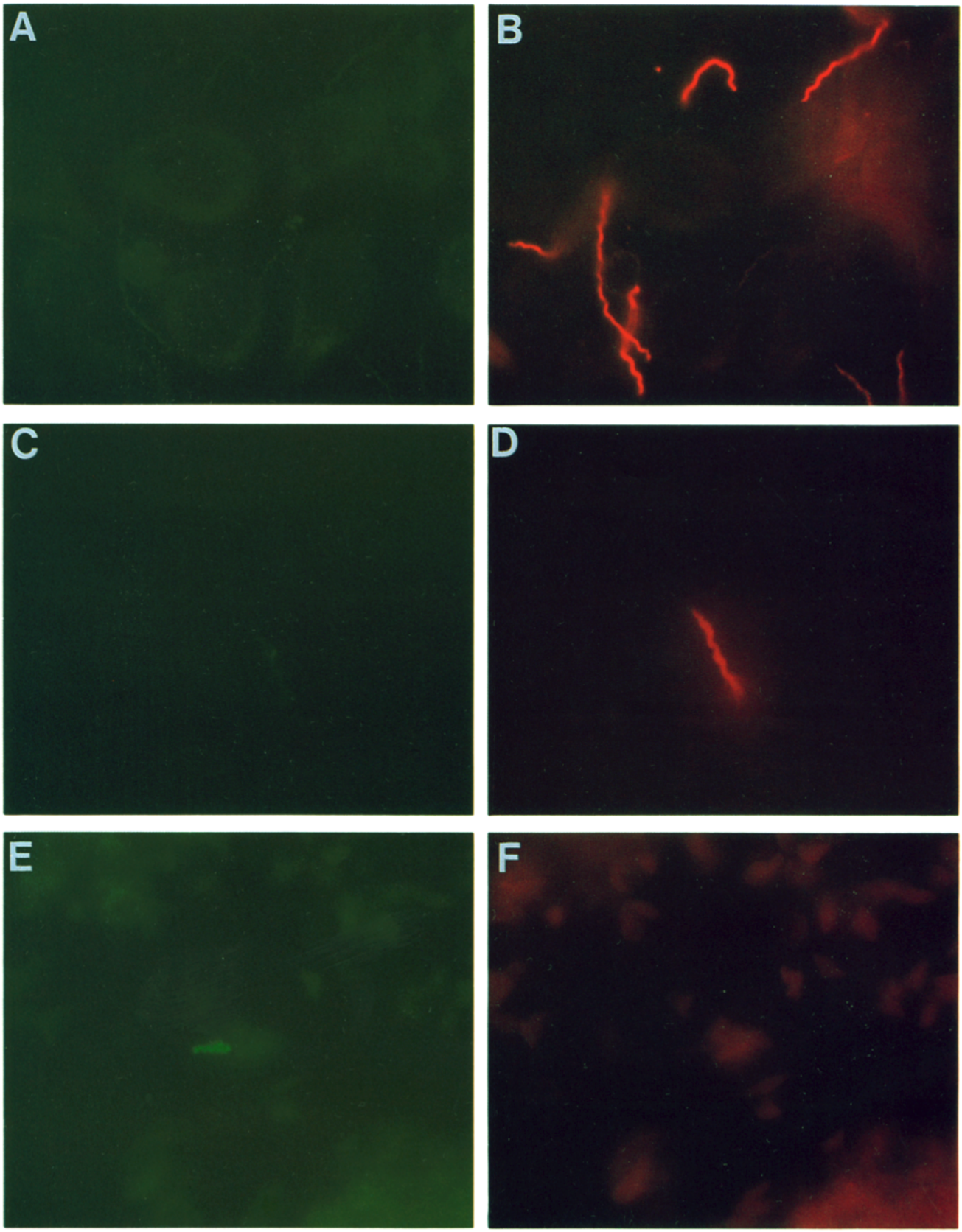

Figure 3. Spirochetes ex vivo can express either Osp A or Osp C. Peritoneal lavage fluids from infected mice were isolated on days 7, 14, and 30 after infection with *B. burgdorferi*. Spirochetes were fixed and double labeled with mouse serum directed against rOsp C and rabbit antisera directed against either Osp A or against cN40 organisms. Labeling was performed both in suspension and on adherent macrophages as described previously. Images were viewed under oil immersion and photographed at ×100. (*A* and *B*) Cultured N40 spirochetes. (*C–F*) Spirochetes recovered from infected animals by peritoneal lavage. FITC (*A*, *C*, and *E*) labels Osp C, and TRITC (*B*, *D*, and *F*) labels Osp A or N40.

ies directed against Osp A (Fig. 3 *B*; TRITC), but had little or no detectable labeling with antibodies directed against Osp C (Fig. 3 *A*; FITC). Taken together, these three methods of evaluating protein expression strongly indicate that Osp A but not Osp C was present at significant levels on this cloned population of spirochetes.

Spirochetes are rarely sighted in an infected animal, and recovery from organs such as skin or heart in sufficient numbers for study is unlikely. In the course of our macrophage studies (27), however, we were able to reculture spirochetes from peritoneal lavage fluids of mice infected for 60 or 90 d after intradermal inoculations of 10^3 or 10^5 cN40 spirochetes. The uninflamed peritoneum provides extensive surface area for a reservoir of spirochetes, and our reculture data suggests that the site may be immunoprivileged, in the sense that resident macrophages in the peritoneal cavity do not completely clear the spirochetes, which they ingest and kill rapidly in vitro (27). We recognized that these results provided us unique access to a source of tissue-adapted spirochetes in their in vivo state. By limiting dilution analysis, we estimated the recovery of spirochetes from lavage fluids of infected mice to be a minimum of 5–10 organisms per animal (n = 5 quantitative recoveries). In this study, spirochetes were recovered directly from lavage fluids in 13 of 14 experiments, and could be detected equally well by immunofluorescence using anti-N40 hyperimmune serum from experiments performed at days 7, 14, and 30 after inoculation.

We have used these organisms to analyze in vivo spirochetal protein expression. Spirochetes were labeled after fixation, either adherent, using peritoneal macrophages to capture spirochetes, or in suspension. Organisms were double labeled to determine protein expression profiles and to garner more information from the limited material. Indirect immunofluorescence of fixed, cultured spirochetes in the presence of macrophages revealed that Osp A expression (Fig. 3 *B*; TRITC) was readily apparent, whereas Osp C staining was at the level of background immunofluorescence (Fig. 3 *A*; FITC). In contrast, some ex vivo spirochetes labeled distinctly for Osp C (Fig. 3 *E*) and not for Osp A (Fig. 3 *F*). Osp A–labeled spirochetes (Fig. 3 *D*) that did not bind Osp C antibodies (Fig. 3 *C*) were also present. These findings were consistent for spirochetes obtained from animals killed at 7 and 14 d after infection (n = 3 each) and show that in vivo, some spirochetes can express either Osp C or Osp A. At day 30 of infection, however, only Osp C–expressing spirochetes were visualized. Staining patterns were identical using different fixation techniques (paraformaldehyde with or without methanol or saponin to permeabilize), whether *B. burgdorferi* were captured on macrophage monolayers or stained in suspension, whether antibodies used were of mouse or rabbit origin, and whether secondary reagents were FITC or TRITC (data not shown). Preincubating cultured spirochetes with either mouse serum or anticoagulated mouse blood before fixation and antibody labeling did not alter our ability to stain with Osp A or N40 antisera, indicating that endogenous mouse antibodies, which could potentially bind to spirochetes in vivo (29), are not likely to contribute to the immunofluorescence observed. Spirochetes cultured from lavage fluid obtained from mice infected for 30 d and regrown to confluence in BSK II medium labeled only with reagents directed against Osp A, not Osp C, corresponding to the phenotype of in vitro cultured spirochetes labeled simultaneously (data not shown).

To determine how the spirochete appears to the immune system in these infected mice, sera were analyzed for the presence of Osp A and C antibodies by immunoblotting rOsp A and C. Weak IgM antibodies to Osp A were greatest at day 28 (Fig. 4 *A*), the latest time point in this experiment, whereas IgG antibodies were at the threshold level of detection. In contrast, IgM and IgG Osp C antibodies were present at day 14 of infection (data not shown) and were strongly represented at day 28 (Fig. 4 *B*). These results support the immunofluorescence data indicating that both Osp A– and Osp C–expressing spirochetes are present at some time before (and, in the case of Osp C, including) day 28 of infection. They also suggest that the late appearance of IgG Osp A antibodies, which occurs both in mice and in humans, may be anamnestic rather than a primary immune response.

To confirm our morphological and immunological obser-

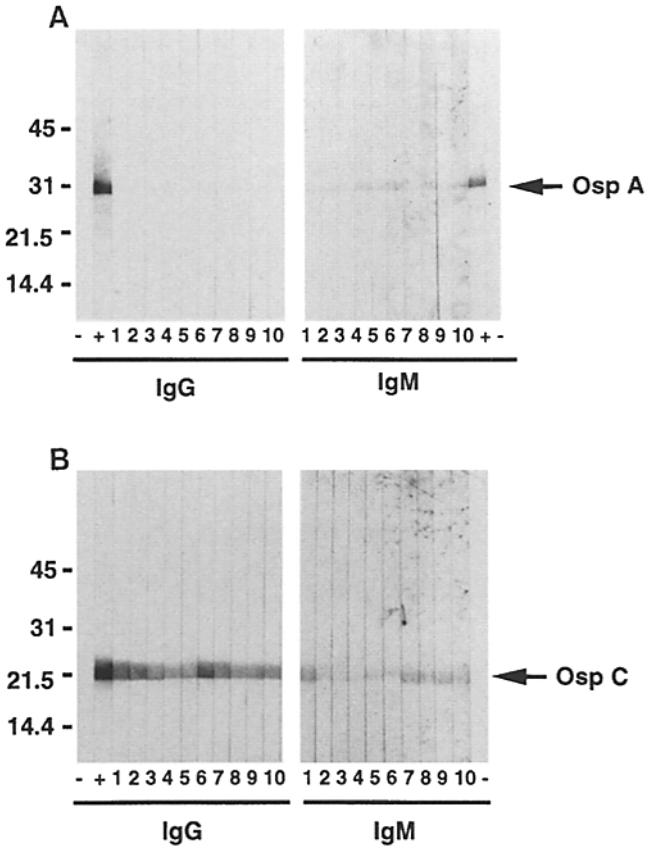

Figure 4. Osp C elicits stronger humoral immunity than does Osp A. Sera from mice killed at 28 d of infection were analyzed for IgM and IgG antibodies to Osp A and Osp C by immunoblot. (*A*) Immunoblot of rOsp A with 1:100 dilution of sera from individual mice (lanes *1–10*) using alkaline phosphatase–conjugated goat anti–mouse IgM- or IgG-specific secondary antibodies. (*B*) Immunoblot of rOsp C using the same sera. Negative and positive controls using NMS (−) and polyclonal anti–Osp A or C antisera (+) are as indicated.

vations, we prepared mRNA from lavage fluid of similarly infected mice and attempted to detect Osp A and C mRNA by RT-PCR, but were hampered by the small numbers of organisms and hence *B. burgdorferi* mRNA recovered, especially in relation to the abundant eukaryotic mRNA. We therefore prepared total RNA from the hearts of mice that were killed 14 and 30 d after inoculation with 10^3 cN40. Hearts were chosen as a source for RNA because spirochetes can be visualized at this site by silver stain during periods of disease activity (30). First-strand DNA was synthesized using random hexamers and 5 μg RNA as template in each 50-μl reaction volume. Mock first-strand reactions were performed in which the reaction components were assembled and incubated in the absence of Superscript RT and then used as templates for PCR to analyze for the presence of contaminating DNA in the RNA preparation. PCR of 10 μl of the first-strand reaction using primers for the eukaryotic RNA actin and *B. burgdorferi*–specific primers for Osps A and C were carried out in an identical fashion as for cultured organisms. Amplified products of the expected size could be detected in both the Osp A (Fig. 5 *A*, lane *5*) and Osp C reactions (Fig. 5 *A*, lane *8*), consistent with the presence of mRNA for these proteins in day-14 infected hearts. RT-PCR of uninfected heart mRNA yielded a product only for actin (data not shown). Whereas Osp C mRNA could be detected in day-30 hearts by this method (Fig. 5 *B*, lane *8*), no amplified Osp A product was visualized by agarose gel electrophoresis under identical conditions (Fig. 5 *B*, lane *5*; $n = 3$). Because the RT-PCR reactions were performed on similar amounts of mRNA derived from hearts at days 14 and 30 of infection, these results strongly suggest that the relative amount of Osp A mRNA has diminished over time.

Taken together, our studies demonstrate that 14 d after syringe inoculation with a clonal population of Osp A^+/Osp C^- spirochetes, both Osp C^+/Osp A^- and Osp A^+/Osp C^- spirochetes are present in the murine host. Because these studies were not performed in a quantitative fashion, we cannot determine the absolute abundance of each type of spirochete. Although it is possible that a rare spirochete within the original inoculum expressed Osp C, the fact that such a spirochete could be visualized ex vivo and that Osp C mRNA could be detected from RNA isolated from infected tissues but not from RNA of cultured organisms indicates that either Osp C is upregulated or that Osp C–expressing spirochetes have a selective growth advantage over Osp A–expressing spirochetes in the mammalian host.

Other investigators have detected Osp C expression on cultured spirochetes, including N40, by immunoblot (16, 18, 19). Several proteins comigrate around the 22-kD region on electrophoresis of spirochetal lysates and using *Borrelia*-infected mouse or human sera to identify their presence does not distinguish reactivity to Osp C with antibodies to other, similarly located proteins on one-dimensional gels. Immunoblotting with antisera produced against rOsp C is a more specific method of detecting the presence of Osp C. Lysates generated from nonclonal populations of spirochetes could contain spirochetes with mixed phenotypes,

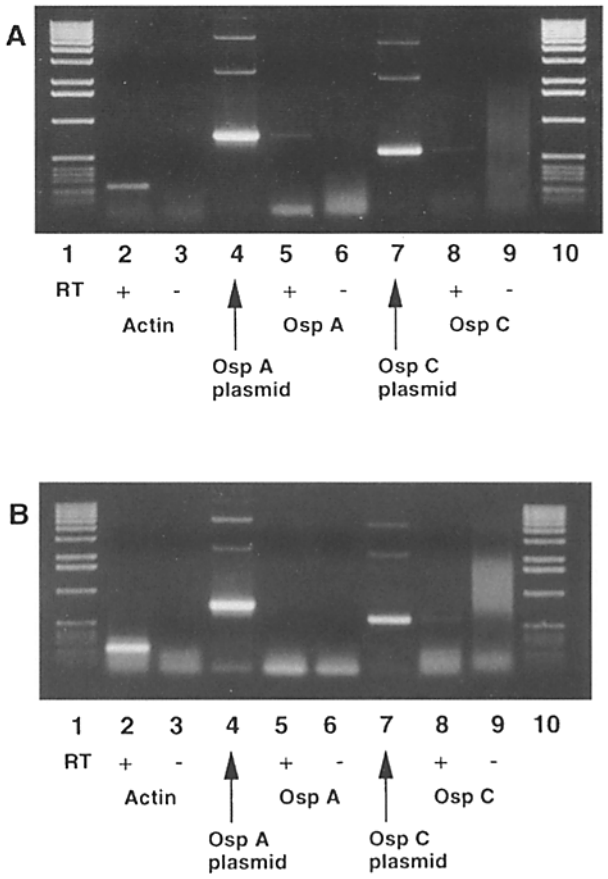

Figure 5. mRNA for Osp C persists for at least 30 d whereas that for Osp A does not. mRNA isolated from infected hearts (*A*, infection day 14, *B*, infection day 30) was used to produce first-strand cDNA using random hexamers as described in Materials and Methods. Actin, Osp A, and Osp C cDNAs were amplified by PCR and analyzed by agarose gel electrophoresis. (Lanes *1* and *10*) 1-kb ladder (Gibco Laboratories, Grand Island, NY). (Lanes *2*, *5*, and *8*) PCR of cDNA using actin- (lane *2*), Osp A- (lane *5*), and Osp C- (lane *8*) specific primers. (Lanes *3*, *6*, and *9*) PCR of mock first-strand reactions using actin- (lane *3*), Osp A- (lane *4*), and Osp C- (lane *5*) specific primers. Positive controls for PCR are products from amplification of Osp A and C genes subcloned into Bluescript II KS (lanes *4* and *6*, respectively). Predicted sizes of amplified products are actin, 245 bp; Osp A, 801 bp; and Osp C, 590 bp.

some of which could express Osp C. In addition, Osp C expression may be stress related (31), so that even clonal cultures grown to late log phase (which simulates a stress condition) could possibly upregulate Osp C. For these reasons, we used a clonal population of N40 in our studies, and lysates for immunoblots were made from a defined number of spirochetes in logarithmic phase of growth. Osp C expression was absent not only by immunoblot, but also by RT-PCR for mRNA and by immunofluorescent staining of fixed spirochetes. Taken together, these three complementary methods, although individually not conclusive, strongly suggest that the spirochetes used in our studies did not express significant amounts of Osp C in culture.

Our results performed in the mammalian host are consistent with those found by Schwan et al. (31) in similar studies carried out in the tick. Infected flat (i.e., unfed) ticks con-

tained spirochetes that were not detected by labeling with antibodies directed against Osp C, but rather were visualized using reagents directed against Osp A. In contrast, after feeding, both Osp A and C expression could be detected on midgut spirochetes. These authors also show that Osp C expression on cultured organisms varied with temperature, with spirochetes grown at 24°C producing no Osp C, whereas those grown at 37°C rapidly upregulate this protein. Spirochetes grown at 37°C were a phenotypically mixed population, with some expressing only Osp A, some only Osp C, and some both proteins. In our studies, spirochetes obtained from peritoneal lavage fluids of infected mice expressed only Osp A or Osp C, but not both. The failure to detect Osp A^+/Osp C^+spirochetes may be due to the relatively low abundance of organisms in the lavage fluid.

The studies reported here do not differentiate between selective pressure for expansion of a particular clone of *B. burgdorferi* and true antigenic variation of the organism after infection. We have therefore referred to our results as antigenic substitution, which covers both possibilities. It has been suggested that Osp C expression may be necessary for infectivity and that exposure to mammalian blood may influence its regulation (32). 14 d after intradermal syringe inoculation of spirochetes into the shoulder region, we found both Osp A^+/Osp C^- and Osp C^+/Osp A^- spirochetes in the peritoneal cavities of infected mice. Presumably, these spirochetes arrived in the peritoneal cavity via a blood-borne route. Unless antigenic variation is occurring, this finding suggests that both types of spirochetes are capable of disseminating from the initial inoculation site and can exist simultaneously at the higher core temperature of the mouse.

We began these investigations to explain why Osp A antibodies are absent or only weakly detected in patients with early Lyme disease. We have shown elsewhere that priming mice with a single Osp A Th cell epitope before infection by syringe inoculation results in earlier IgG seroconversion to Osp A than would normally occur during infection (Bockenstedt, L. K., E. Fikrig, S. W. Barthold, R. A. Flavell, and F. S. Kantor, manuscript in preparation). When seroconversion occurred, it could be detected within the first 2 wk of infection; longer experimental periods did not result in greater numbers of animals seroconverting to Osp A. These findings suggest that the amount of Osp A present within the first several days of infection is a critical determinant of IgG antibody production, and they also indicate that Osp A antigen becomes limiting relatively soon after infection. In the current studies, we were able to detect Osp A on spirochetes both by immunofluorescence and at the mRNA level at day 14, but not at day 30, of infection. The weak antibody response may therefore result, in part, from the transient expression of Osp A by spirochetes in the infected host. Downregulation of Osp A after infection is consistent with studies by Barthold et al. (16), in which mice immunized with Osp A were not protected from challenge infection by transplantation of skin from syngeneic mice previously infected with 10^4 *B. burgdorferi* strain N40. In the same studies, the authors show that mice inoculated with 10^7 heat-killed N40 seroconvert to Osp A, but not to 22-kD proteins, including Osp C. In contrast, mice inoculated with as few as 10^1 viable organisms seroconvert to Osp C (as well as other 22-kD proteins), but not to Osp A. Our analysis of Osp expression on tissue-adapted spirochetes at both the protein and mRNA level support these immunologic data, indicating that Osp C is expressed on spirochetes after infection and that Osp A^+ spirochetes become less readily apparent over time.

To ensure its continued survival in nature, *B. burgdorferi* must adapt readily to the disparate environments of its two major host reservoirs, ticks and mammals. One way in which *B. burgdorferi* may accomplish this is to vary its expression of characteristic surface proteins. Variation of Osps by infectious agents is well documented both in spirochetes and other microorganisms (32). For example, the related spirochete, *Borrelia hermsii*, the causative agent of relapsing fever, varies its major surface protein by gene rearrangements (33), and *Serpulina hyodysenteriae* has strain-specific expression of an important virulence determinant (34). In *B. burgdorferi*, variations in expression are known at the mRNA level (35) and protein level (36) and after prolonged in vitro culture (37). Other possible mechanisms for changing protein expression are downregulation or shedding of characteristic proteins in vivo, or covering characteristic proteins with a "slime layer" of spirochete or of host origin (30, 38).

Taken together, the results presented in this study demonstrate that C3H mice infected by syringe inoculation with *B. burgdorferi* strain N40 spirochetes expressing only Osp A are exposed to and mount an early immune response to both Osp A and Osp C, although the former protein elicits a much weaker response. Our investigations indicate that Osp A expression diminishes during early infection, providing one explanation for the weaker antibody response to this protein. Moreover, our close study of spirochetes ex vivo is the first indication that Osp C–expressing spirochetes expand preferentially over those expressing Osp A in the mammalian host. These findings suggest a previously unrecognized role for Osp C in spirochetal adaptation and survival in the mammalian host.

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