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Circumvention of Outer Surface Protein A Immunity by Host-Adapted *Borrelia burgdorferi*

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Outer surface protein A (OspA), which is abundantly expressed in cultured *Borrelia burgdorferi*, appears to be down-regulated or masked following low-dose infection, and OspA immunization did not prevent infection, dissemination, or disease development with host-adapted spirochetes. Seroconversion of mice to *B. burgdorferi* OspA depended on dose and viability of inoculated spirochetes. Mice inoculated with $>10^4$ live spirochetes and $>10^7$ heat-killed spirochetes seroconverted to OspA, but mice inoculated with fewer spirochetes did not seroconvert to OspA at 2 weeks after inoculation. Growth temperature of spirochetes was not a factor for infectious dose or seroconversion to OspA. Spirochetes grown at 30, 34, or 38°C had the same median infectious dose. Growth temperature did not influence infectious dose when mice were inoculated intraperitoneally or intradermally and did not influence dose-related immunologic recognition of OspA. Mice hyperimmunized with recombinant OspA–glutathione *S*-transferase (GT) fusion protein or GT (controls) were challenged by syringe inoculation with 10^3 spirochetes or by transplantation of infected skin from syngeneic mice infected for 2 or 8 weeks. OspA-GT-immunized mice resisted syringe challenge but developed disseminated infections following transplantation of infected skin. Identical results were obtained in mice passively immunized with hyperimmune serum to OspA-GT or GT and then challenged by syringe or infected skin transplant. The number of spirochetes in infected skin, determined by quantitative PCR directed toward both plasmid and genomic targets, was less than the syringe challenge dose.

Borrelia burgdorferi, the agent of Lyme disease, expresses abundant outer surface protein A (OspA), a species-specific lipoprotein (6) which, along with flagellin, accounts for approximately one-third of the total spirochete protein (15). Paradoxically, human patients with naturally acquired Lyme disease may not seroconvert to OspA, may seroconvert with low antibody titers, or may seroconvert only after many months of infection (1, 4, 19, 31, 33, 54), despite the fact that recombinant OspA is immunogenic in humans (32, 52). The lack of OspA immune recognition during naturally acquired infection is not unique to humans, as it has become apparent that rhesus monkeys, dogs, mice, and hamsters with tick-borne infection do not seroconvert to OspA, whereas they do following syringe inoculation (2, 27–29, 37–39). These observations suggest that cultured spirochetes may differ from tick-borne spirochetes in OspA expression, but both cultured spirochetes and spirochetes within the midguts of infected ticks have been shown to label with antibody to OspA (6, 22), and OspA-induced immunity in laboratory rodents is protective against both syringe- and tick-borne *B. burgdorferi* infection (22–24, 26, 40, 41, 46, 50). The difference in OspA immune recognition between syringe- and tick-borne inoculation may simply be the result of dose rather than source of the spirochetes, as laboratory mice infected intradermally with low doses of spirochetes do not seroconvert to OspA until late in the course of infection (8, 9, 26), as seen in naturally infected humans (1, 4, 19, 31, 33, 54).

These observations suggest that *B. burgdorferi* expresses OspA in culture and in ticks, but OspA may not be expressed or is masked upon adaptation to the mammalian host, thus eliciting no significant or discernible antibody response, at least in the early months following low-dose infection, despite the fact that other, less abundant proteins are immunologically recognized. The purpose of this study was to determine if OspA becomes occult upon adaptation to the mammalian host, and if so, prove that immunity to OspA, which is protective against syringe-borne *B. burgdorferi* challenge, is irrelevant against host-adapted *B. burgdorferi*.

MATERIALS AND METHODS

Mice. C3H/HeNCrlBR (C3H) mice were purchased from Charles River Laboratories, Raleigh, N.C. Mice were shipped in filtered crates, maintained in isolator cages (Lab Products, Maywood, N.J.), and provided food (Agway, Syracuse, N.Y.) and water ad libitum. They were killed with carbon dioxide gas and then exsanguinated by cardiocentesis.

B. burgdorferi. A clonal population of the *B. burgdorferi* N40, with proven infectivity and pathogenicity in laboratory mice (9), was used to inoculate mice and as the antigen for immunoblots. Spirochetes were grown in modified Barbour-Stoenner-Kelly (BSKII) medium (5) at 34°C and, in one experiment, at 30 or 38°C. Spirochetes for inoculation were grown to log phase and enumerated in a Petroff-Hausser bacterial counting chamber (Baxter Scientific Products, McGaw Park, Ill.). Heat killing was accomplished by incubation of culture tubes in a 60°C water bath for 1 h. Killing was verified by culture of aliquots from the heat-treated cultures and culture of tissues from inoculated mice. Infection of inoculated mice was determined by culture of blood, spleen, urinary bladder, and ear punches as described previously (9). Cultures were incubated for 2 weeks and then examined for spirochetes by dark-field microscopy to determine growth.

Immunoblots. *B. burgdorferi* proteins and recombinant proteins OspA, NS1-P39, flagellin, and OspC (see below) were transferred from sodium dodecyl sulfate-polyacrylamide gels to nitrocellulose membranes as described previously (8), using a Hoefer Transphor cell and power lid (Hoefer Scientific Instruments, San Francisco, Calif.). Nitrocellulose paper was cut into strips, blocked with calf serum, incubated with 1:100 dilutions of test sera overnight in a refrigerator, incubated with goat anti-mouse immunoglobulin M (IgM) or IgG (Kirkegaard + Perry Laboratories, Inc., Gaithersburg, Md.), and then stained with a 5-bromo-4-chloro-3-indolylphosphate/nitroblue tetrazolium phosphatase substrate system (Kirkegaard + Perry Laboratories) as detailed elsewhere (8). A number of antibodies, including antibodies to OspA, P39, flagellin, and OspC (see below), served as reference markers for selected *B. burgdorferi* proteins.

Antisera. These studies used monoclonal antibodies to OspA (C3.78) and

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41-kDa flagellin (H11.61), provided by F. S. Kantor, and polyclonal antibodies generated by hyperimmunization of mice with recombinant fusion proteins NS1-P39, OspA–glutathione *S*-transferase (GT), flagellin-GT, OspC-GT, and a soluble truncated OspA (see below).

Recombinant proteins. NS1-P39 fusion protein, containing the influenza virus nonstructural protein NS1 and *B. burgdorferi* P39 elements (26, 29, 39, 47), was provided by Y. Lobet, SmithKline Beecham Biologicals, Rixensart, Belgium. OspA-GT, flagellin-GT, and OspC-GT fusion proteins, all derived from the *B. burgdorferi* N40 DNA, were prepared and purified as previously described (22, 23, 48). A soluble truncated OspA, in which the signal sequence (first 17 amino acids) was replaced by methionine-alanine, was generated and purified from *B. burgdorferi* N40 DNA as described previously (10, 20). Immunoblots were prepared with NS1-P39, truncated OspA, flagellin-GT, GT, and OspC following cleavage of its GT fusion partner with thrombin.

Histology and immunohistochemistry. Rear legs and hearts were immersion fixed in formalin, demineralized, embedded, sectioned, and stained with hematoxylin and eosin (8). Joints (both knees and tibiotarsi) and hearts were evaluated blindly and scored for the presence and severity of inflammation as described previously (11). Indirect immunohistochemistry was performed on archival arthritic rear legs from C3H severe combined immunodeficient (SCID) mice infected with *B. burgdorferi* (11), or on the rear leg from a C3H mouse, 1 h after inoculation of the footpad with 10^8 spirochetes. Primary sera consisted of 1:10 dilutions of hyperimmune serum to OspA-GT and serum from mice infected for 90 days following intradermal inoculation with 10^3 spirochetes. Tissues were formalin fixed, paraffin embedded, sectioned, bonded to glass slides, trypsinized, incubated with 1:10 dilutions of primary sera, and labeled with a biotinylated goat anti-mouse IgG/streptavidin-peroxidase method as described previously (3).

Immunization and challenge. Mice were immunized with 5 μg of OspA-GT or GT subcutaneously in complete Freund's adjuvant and then given two booster injections with the same amount of protein in incomplete Freund's adjuvant 2 and 4 weeks after the initial immunization. Immunization against OspA was verified by immunoblot reactivity to OspA at serum dilutions of at least 1:10,000 in all OspA-vaccinated mice. Vaccinated mice were then challenged intradermally with 10^3 *B. burgdorferi* spirochetes or challenged by transplantation of two 1.5-mm-diameter pieces of ear from donor mice that had been infected for periods of 2 or 8 weeks. Transplant donor mice were inoculated intradermally with 10^4 *B. burgdorferi* spirochetes 2 or 8 weeks prior to transplantation of tissues into vaccinated, recipient mice. On the day of transplantation, donor mice were killed, and ear pinnae were disinfected with alcohol, excised, placed in warm BSKII medium, and diced into 1.5-mm pieces. Ear samples from each donor mouse, as well as urinary bladder and spleen, were cultured to verify infection of donor mice, and two ear pieces were transplanted subcutaneously into each recipient mouse as previously described (7). Each recipient mouse received transplants from a separate donor mouse. All donor mice and all recipient mice were verified to be infected on the basis of culture. Vaccinations, inoculations, and transplants were coordinated so that all mice were challenged on the same day. Mice were killed and examined for infection and disease at 2 weeks after challenge.

Mice were also passively immunized with hyperimmune serum to OspA-GT or GT, obtained from mice subjected to the same immunization protocol and verification of antibody titers outlined above. Naive mice were passively immunized subcutaneously with 0.5 ml of 1:10-diluted hyperimmune serum and then challenged subdermally 18 h later with 10^3 *B. burgdorferi* spirochetes, 10^3 *B. burgdorferi* spirochetes plus transplanted normal ear pieces, or transplanted ear pieces from mice infected for 2 weeks as described above. Mice were killed and examined for infection and disease at 2 weeks after challenge.

Quantitative analysis of *B. burgdorferi* in tissue. Full-thickness 1.5-mm-diameter ear punches were collected under strict aseptic technique to minimize both bacterial and DNA contamination (35) from five mice at 15 days and five mice at 90 days after intradermal inoculation of a distant site (shoulder region) with 10^3 *B. burgdorferi* spirochetes. A 50-μl digest was obtained from each piece of tissue; 2- or 5-μl aliquots were then twofold serially diluted to 1:256, and each dilution was tested for *B. burgdorferi* plasmid and chromosomal targets, using *ospA* or flagellin gene primers (36). Previous analyses in our laboratory have shown this method to accurately estimate numbers of spirochetes present in limiting dilution series and to be comparable to competitive template PCR methods for the purposes of quantification of *B. burgdorferi*. The number of spirochetes per standard-size ear punch from each mouse was estimated on the basis of the most conservative assumptions of a single genome copy per spirochete (30) and single-copy sensitivity of the terminal dilution.

RESULTS

We initially sought to discriminate between the antibody response to *B. burgdorferi* antigens expressed during active infection and responses that were recognized simply as a result of antigenic stimulation of the original inoculum (reflecting antigens expressed in vitro). To do so, we inoculated mice with diminishing numbers of live or heat-killed *B. burgdorferi*. A 7-ml culture containing log-phase *B. burgdorferi* was enumerated and serially diluted with BSKII medium; then each serial dilution was split, and one half of each dilution was heat killed. Groups of two mice each were inoculated intradermally with 10^7, 10^6, 10^4, 10^2, or 10^1 live or dead spirochetes in 0.1 ml. At 14 days after inoculation, mice were killed, and blood, spleen, and bladder were cultured to verify infection status. None of the mice inoculated with killed spirochetes became infected. Infection could be verified in all mice inoculated with 10^7 through 10^4 spirochetes, one of the two mice inoculated with 10^2 spirochetes, and neither of the two mice inoculated with 10^1 spirochetes. IgM and IgG immunoblots were prepared on sera from each mouse, and reactivities of antibodies to proteins were tabulated (Table 1). IgM responses of all mice were similar to IgG responses except that all mice, including uninoculated control mice, had IgM reactivity to a 41-kDa protein, presumed to be flagellin. Mice inoculated with 10^7 heat-killed spirochetes had IgG reactivity to 31-, 34-, and 41-kDa proteins, whereas mice inoculated with the same dose of viable spirochetes also developed reactivity to 39- and 22-kDa proteins, as well as a few other minor higher- and lower-molecular-weight bands (Fig. 1). The locations and presumed identities of these principal immunoreactive proteins were determined in Western blots (immunoblots) incubated with monoclonal antibodies to OspA and flagellin and polyclonal antibodies to P39 and OspC. Reactivity of sera from mice inoculated with heat-killed spirochetes diminished markedly at lower doses, with reactivity restricted to the 41-kDa protein at the 10^6 dose but not at doses of 10^4 or below. In contrast, mice inoculated with diminishing doses of viable spirochetes maintained strong reactivity to 22-, 39-, and 41-kDa proteins at all doses. Reactivity to OspA and OspB disappeared at doses of 10^6 or below, but there was reactivity to an approximately 32- or 33-kDa band, which did not align with OspA or OspB. Sera from three of the four mice inoculated with 10^2 or 10^1 viable spirochetes reacted strongly to 22-, 39-, and 41-kDa proteins and weakly to a narrow 32- to 33-kDa band (Fig. 2), but reactivity to other proteins, including OspA, was absent. In addition, sera from mice with tick-borne infection from another study (50) were tested and had immunoblot reactivity similar to that of low-dose-infected mouse sera. Absence of serum reactivity to OspA and positive reactivity to flagellin, P39, and OspC proteins were verified by performing immunoblots with low-dose (10^1 and 10^2)-infected mouse sera against these recombinant proteins.

In an effort to determine if a shift in temperature from culture (34°C) to body temperature (core temperature of 38°C) may be a factor in modifying OspA expression and serological response, culture tubes were inoculated with *B. burgdorferi* and then grown to log phase for 10 days at 30, 34, or 38°C. To determine if skin (having a cooler surface temperature) was a factor in *B. burgdorferi* adaptive OspA expression or immune recognition, the serological response of mice inoculated intradermally was compared with that of mice inoculated intraperitoneally, in which the environment would approach core body temperature. Paired groups of three mice were inoculated intradermally or intraperitoneally with 10^5, 10^4, 10^3, 10^2, or 10^1 spirochetes. Mice were killed at 14 days, and infection was verified by culture of spleen and blood. In this experiment, the median infectious doses were compared among mice inoculated with spirochetes grown at different temperatures and given by different routes (Table 2). Temperature did not affect the infectious dose, although the route of inoculation was critical, as intradermally inoculated mice could be infected with 10-fold-fewer spirochetes compared with intraperitoneally inoculated mice. Temperature or route of inoculation did not

TABLE 1. Immunoblot reactivity of sera to *B. burgdorferi* proteins at 14 days after intradermal inoculation of mice with different doses of heat-killed (dead) or live *B. burgdorferi*

Dose	Live or dead	Ig	Culture[a,b]	Reactivity to protein[a]					
				<15 kDa	22 kDa	31 kDa	34 kDa	39 kDa	41 kDa
10^7	Dead	IgM	−, −	−, −	+, +	+, +	−, −	−, −	+, +
10^6	Dead	IgM	−, −	−, −	−, −	−, −	−, −	−, −	+, +
10^4	Dead	IgM	−, −	−, −	+, −	−, −	−, −	−, −	+, +
10^2	Dead	IgM	−, −	−, −	−, −	−, −	−, −	−, −	+, +
10^1	Dead	IgM	−, −	−, −	−, −	−, −	−, −	−, −	+, +
10^7	Live	IgM	+, +	−, −	+, +	+, +	+, −	+, +	+, +
10^6	Live	IgM	+, +	−, −	+, +	+, *	+, −	+, +	+, +
10^4	Live	IgM	+, +	−, −	+, +	*, *	−, −	+, +	+, +
10^2	Live	IgM	−, +	−, −	+, +	−, −	−, −	+, +	+, +
10^1	Live	IgM	−, −	−, −	−, +	−, −	−, −	−, +	+, +
10^7	Dead	IgG	−, −	−, −	−, +	+, +	+, −	−, −	+, +
10^6	Dead	IgG	−, −	−, −	−, +	−, −	−, −	−, −	+, +
10^4	Dead	IgG	−, −	−, −	−, −	−, −	−, −	−, −	−, −
10^2	Dead	IgG	−, −	−, −	−, −	−, −	−, −	−, −	+, −
10^1	Dead	IgG	−, −	−, −	−, −	−, −	−, −	−, −	−, −
10^7	Live	IgG	+, +	+, +	+, +	+, +	+, +	+, +	+, +
10^6	Live	IgG	+, +	+, +	+, +	*, +	−, +	+, +	+, +
10^4	Live	IgG	+, +	+, +	+, +	*, *	−, −	+, +	+, +
10^2	Live	IgG	−, +	−, −	+, +	*, *	−, −	+, +	+, +
10^1	Live	IgG	−, −	−, −	−, +	−, *	−, −	−, +	−, +
0 (control [normal])		IgM	−, −	−, −	−, −	−, −	−, −	−, −	+, +
		IgG	−, −	−, −	−, −	−, −	−, −	−, −	−, −

[a] Results for mouse 1 and mouse 2, respectively. * denotes reaction to a narrow 32-kDa band which may be obscured by the wider 31-kDa (OspA) band at higher doses.

[b] *B. burgdorferi* cultured from blood, spleen, and/or urinary bladder.

influence OspA immune recognition, but a dose-related effect was seen with OspA reactivity, as in the previous experiment.

Next, the role of OspA immunity in preventing infection with host-adapted spirochetes was explored. Both passive and active OspA immunization effectively prevent syringe- and tick-borne challenge infection in mice (22–24, 26, 40, 41, 46, 50), but if OspA expression is ameliorated or masked once spirochetes have adapted to the host, or spirochetes are inaccessible to OspA immune responses, OspA immunity should be ineffective at preventing infection with host-adapted spirochetes. To test this hypothesis, mice were immunized with OspA-GT or GT and then syringe challenged intradermally with 10^3 *B. burgdorferi* spirochetes or challenge infected by transplantation of tissue from infected mice. Since the duration of infection may have an effect on OspA expression, tissues for transplant challenge were obtained from mice at 2 weeks, representing peak disease, and at 8 weeks, representing the immune-mediated disease resolution phase of infection (8, 9). Mice were killed at 2 weeks after challenge. OspA-GT-immunized mice were resistant and GT-immunized mice were susceptible to syringe challenge, as expected (Table 3). Mice immunized with OspA-GT or GT were both susceptible to infection following transplantation of ear pieces from infected mice. Furthermore, all mice infected by tissue transplantation developed polysynovitis and carditis, with no differences in disease prevalence or severity between OspA-GT and GT immunization. No differences were found between mice infected by tissue from donors infected for 2 or 8 weeks. Serum from transplant-infected, control mice reacted to 22-, 39-, and 41-kDa proteins, but not OspA, on immunoblots.

These findings were confirmed with passive immunization experiments (Table 4). We also tested for the effect of transplanting normal skin in the region of the syringe inoculum. Groups of four naive mice were given 0.5 ml of 1:10 dilutions of OspA-GT or GT hyperimmune serum subcutaneously and then challenged subdermally 18 h later with 10^3 spirochetes, 10^3 spirochetes plus normal skin pieces, or skin pieces from infected mice. Results were similar to those for the active immunization experiment: mice passively immunized with OspA-GT antiserum resisted syringe challenge (with or without normal ear pieces) but were fully susceptible to both infection and disease following challenge infection by transplantation of infected ear pieces.

Since OspA-induced immunity can be overcome with high syringe doses of spirochetes (23), differences in protective immunity noted between syringe- and transplant-borne infection could be a function of spirochete dose. We therefore quantified the number of spirochetes in standard-size ear punches (equivalent to the size of tissue used in the transplant challenge) from mice infected with the same dose of spirochetes used to inoculate the transplant donor mice and at 15 and 90 days, intervals that spanned the transplant donor intervals used (Table 5; Fig. 3). As previously noted (36), there was an excess of plasmid target (*ospA*) over chromosomal target (flagellin gene) relative to the 1:1 proportions of these targets found in cultured organisms, but the two targets yielded similar quantitative data. For the sake of comparison, the maximal possible number of spirochetes within a total sample was calculated on the basis of the most conservative assumptions of a single genome copy per spirochete and single-copy sensitivity at terminal dilutions. Excepting a single sample with a calculated 3,200 spirochetes, as judged from a 2-μl input volume and

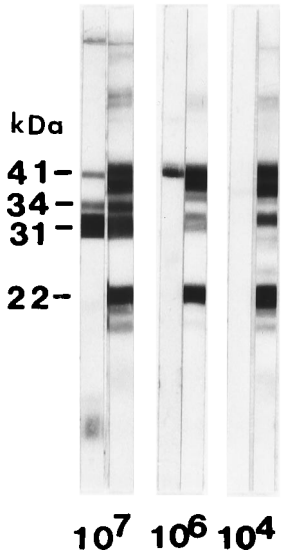

FIG. 1. Representative IgG Western blots (*B. burgdorferi* antigen) of sera from paired mice, 14 days after intradermal inoculation with 10^7, 10^6, or 10^4 heat-killed (left blot of each pair) or live (right blot of each pair) spirochetes. Mice inoculated with live spirochetes seroconverted to 22- and 39-kDa proteins, which was not seen in mice inoculated with heat-killed spirochetes. Antibody to OspA was present only in mice inoculated with the highest dose of killed or viable spirochetes.

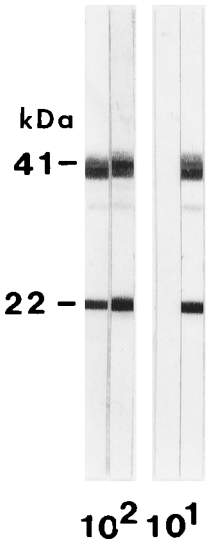

FIG. 2. Western blots (*B. burgdorferi* antigen) of sera from two mice each, 14 days after intradermal inoculation with 10^2 or 10^1 live spirochetes. One mouse inoculated with 10^1 spirochetes presumably did not become infected. The remaining mice seroconverted strongly to 22-, 39-, and 41-kDa proteins, which were verified to be OspC, P39, and flagellin with recombinant proteins. No reactivity to OspA (native or recombinant) could be detected.

flagellin gene target, the maximum estimated number of spirochetes in a standard-size ear punch was calculated to be ≤640. Excepting the single sample, the number of spirochetes in the tissues used for transplant challenge were likely to be well below the 10^3 syringe challenge dose, even under the assumption of only one genome copy of the more abundant *ospA* target per spirochete. Furthermore, the single exceptionally high sample was in the same range (3×10^3) as the syringe dose (10^3).

Collectively, these data implicate a process in which OspA is down-regulated or masked during in vivo adaptation of *B. burgdorferi*. This possibility was directly analyzed by performing immunohistochemistry on tissues from infected mice. Tissues from SCID mice contain spirochetes unencumbered by immune pressures. Serum from C3H mice, collected at 90 days after low-dose (10^3) intradermal inoculation, reacted strongly against *B. burgdorferi* in tissues from SCID mice at a dilution of 1:10 (*B. burgdorferi* immunoblot titer of <1:200), whereas OspA hyperimmune serum, having a *B. burgdorferi* immunoblot titer of >1:100,000 and used at a 1:10 working dilution for immunohistochemistry, did not react against spirochetes in an adjacent serial section from the same mouse (Fig. 4). To ensure validity of the reactions, tissue sections were examined blindly with randomly selected serial sections and repeated with tissues from a second mouse. On the other hand, the OspA antiserum (1:100) reacted with spirochetes which had been inoculated into the footpad of a mouse 1 h previously and were thus not yet host adapted.

DISCUSSION

The dose-related response to OspA noted in this study confirms and extends our previous observations (8, 9) and those of others (26). When inoculated by syringe with low doses of spirochetes, mice do not seroconvert to OspA until several months after infection, like naturally infected humans and dogs (1, 4, 10, 19, 31, 33, 54), as well as rhesus monkeys, dogs, mice, and hamsters experimentally infected by tick-borne inoculation (2, 27–29, 37–39). Thus, the OspA response is dose dependent and not solely a factor determined by tick-borne infection. The OspA response, however, is not simply dependent on the number of spirochetes initially introduced, as it took more heat-killed spirochetes than live spirochetes to elicit an OspA response. This finding suggests that live spirochetes may be more immunogenic because they replicate after inoculation, thereby presenting (albeit transiently) more OspA to stimulate a detectable antibody response before OspA is down-regulated or masked during adaptation to the host. Low doses of viable spirochetes, which do not elicit a detectable OspA response, may remain localized for longer periods before dissemination, allowing OspA down-regulation before significantly immunogenic numbers are available for immune recognition. Regardless, circumvention of the host adaptation period by transplantation of tissue containing adapted spirochetes results in both dissemination and disease without a detectable OspA antibody response.

Mice, like humans, may eventually seroconvert to OspA after several months of infection (1, 4, 9, 19, 31, 33, 54). Recently, seemingly conflicting results have been published: humans in the early stages (erythema migrans) of Lyme disease were found to possess both IgM and IgG antibody to OspA, but such antibody could be detected only with more sensitive biotin-avidin labeling of immunoblots and in the form of im-

TABLE 2. Relative median infectious doses of *B. burgdorferi* grown at different incubation temperatures and inoculated intradermally or intraperitoneally into C3H mice

Growth temp (°C)	Log_{10} spirochetes	
	Intradermal	Intraperitoneal
30	1.8	2.4
34	1.3	2.5
38	1.2	2.4

TABLE 3. Challenge of actively OspA-immunized and control mice with syringe (cultured)- or transplant (host adapted)-borne infection with *B. burgdorferi*

Immunogen	Challenge	Infection rate[a]
OspA-GT	10^3 spirochetes, syringe	0/5
GT		3/3
OspA-GT	Tissue transplant, 2-week-infected donor	5/5
GT		5/5
OspA-GT	Tissue transplant, 8-week-infected donor	5/5
GT		4/4

[a] Number of culture-positive (blood, urinary bladder, spleen, and/or ear) mice/number tested.

mune complexes (43). We did not attempt to use more sensitive detection methods or examine immune complexes, as the results of both studies clearly indicate that antibody to OspA is disproportionately low relative to the amount of OspA that should be represented if it were expressed in vivo to the degree that it is represented in vitro.

The circumstances surrounding host adaptation of *B. burgdorferi*, and in particular expression of OspA, have yet to be clarified but will eventually lead to important new insights into the pathobiology of this organism. The genome of *B. burgdorferi* is composed of one linear chromosome with additional linear plasmids (one containing the *ospA* gene) and several supercoiled circular plasmids, a genetic arrangement that is unique among other bacteria in general and spirochetes in particular (12). During growth in culture, and especially after addition of specific antiserum, *B. burgdorferi* cells actively shed membrane-bound vesicles which contain plasmid constituents, but not chromosomal elements, along with multiple proteins including OspA (25) which bind IgM nonspecifically (17). Furthermore, extracellular blebbing appears to be an event that takes place in vivo, with these structures, complexed with IgM, detectable in a variety of tissues and body fluids (18). This may be one explanation for the target imbalance found in synovial specimens from Lyme arthritis patients, in which there is an excess of plasmid (including *ospA*) target over chromosomal target, while such a discrepancy in target balance is not found among cultured spirochetes (36). We likewise found an overabundance of *ospA* target over flagellin gene target in the ear samples of infected mice.

The OspA adaptive response, whatever its mechanism, does not appear to be the simple effect of transition into a medium (the host) of a different temperature relative to tick or culture,

TABLE 4. Challenge of passively OspA-immunized and control mice with syringe (cultured)- or transplant (host-adapted)-borne infection with *B. burgdorferi*

Immunogen	Challenge	Infection rate[a]	Disease rate[b]
OspA-GT	10^3 spirochetes, syringe	0	0
GT		4	4
OspA-GT	10^3 spirochetes, syringe + normal tissue transplant	0	0
GT		4	4
OspA-GT	Tissue transplant, 2-week-infected donor	4	4
GT		4	4

[a] Number of culture-positive (blood, urinary bladder, spleen, and/or ear) mice out of four tested.

[b] Number of mice with arthritis and carditis out of four examined.

TABLE 5. Quantification of *B. burgdorferi* in ear punches from two groups of five mice each infected for 2 or 8 weeks, using PCR amplification of serial twofold dilutions of samples, different gene targets, and different volumes of target

Interval (wk)	Target	Input vol (μl)	Range, reciprocal endpoint dilution	Estimated no. of spirochetes/ear punch[a]
2	Flagellin gene	2	0–4	0–100
	ospA	2	2–8	5–200
	ospA	5	8–32	80–320
12	Flagellin gene	2	1–128[b]	2–3,200[b]
	ospA	2	2–8	5–200
	ospA	5	16–64	160–640

[a] Maximum estimates based on assumption of one copy of targeted gene per spirochete and single-copy sensitivity at terminal dilutions, calculated for 50 μl of total digest per ear punch.

[b] One sample tested at 128; the other four samples were in the range of 1 to 4.

and entry into skin does not appear to be the critical factor. Spirochetes grown at different temperatures had the same infectious dose and elicited the same dose-dependent OspA response, and the same dose-dependent OspA response was noted in mice regardless of route of inoculation. Others have found no difference in OspA expression during growth at different temperatures, but other proteins, including P39 and OspC, do seem to vary in response to growth temperature (44, 45). Recent studies have shown that *B. burgdorferi* produces OspC in response to both incubation temperature and tick feeding. Spirochetes expressed OspC when incubated at 32 to 37°C or within ticks that had taken a blood meal but not when incubated at 24°C (lower than our lowest temperature of 30°C) or in unfed ticks. Such absolute results were not found with OspA expression, although some spirochetes within the midguts of fed ticks did not express OspA (44). In addition, others have demonstrated a quantitative reduction in OspA expression in ticks following feeding, using an antigen capture enzyme-linked immunosorbent assay (13). Collectively, these studies suggest that *B. burgdorferi* may begin its adaptive response within the feeding tick.

Passively transferred OspA-specific antibody and active OspA immunity have been shown to be highly effective at preventing both syringe- and tick-borne infection in laboratory mice (22, 50, 51). This would be expected, as cultured *B. burgdorferi* and spirochetes in the midguts of ticks express OspA (24, 44), thus making them vulnerable to OspA antibodies before entry into the host. Consistent with this hypothesis, spirochetes are killed directly in the midgut of ticks feeding upon OspA-vaccinated mice (24). Although OspA may be

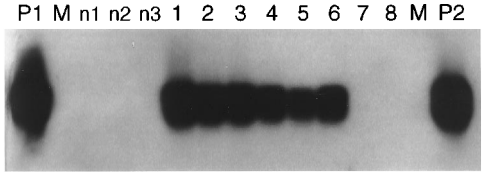

FIG. 3. Representative analysis of terminal-dilution PCR. Twofold serial dilutions of 5 μl of digested ear tissue from an infected mouse were amplified by PCR, using *ospA* PCR primers. On the basis of conservative assumptions of one *ospA* gene target per spirochete and single-copy sensitivity of the primer pair at terminal dilutions, the number of spirochetes estimated to be present in the total 50-μl digest of this specimen was 640. P1, high-positive control; P2 low-positive control; n1 to n3 negative (no target) controls; M, molecular weight markers; lanes 1 to 8, 1:2 through 1:256 dilutions of a 5-μl digest.

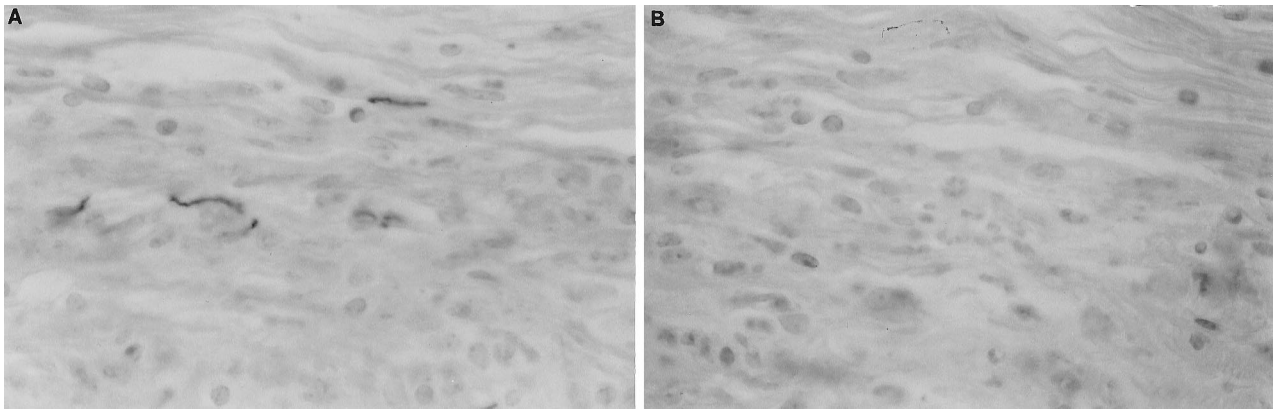

FIG. 4. (A) Spirochetes in joint tissue of a SCID mouse labeled with serum from an immunocompetent mouse, 90 days after inoculation with 10^3 spirochetes; indirect immunoperoxidase. (B) Adjacent section of joint tissue of a SCID mouse labeled with hyperimmune serum to OspA; indirect immunoperoxidase. No labeling of spirochetes was found.

down-regulated or masked, other *B. burgdorferi* proteins, expressed during active infection, are strongly immunogenic. Indeed, very small amounts of OspA antibody-negative serum from mice infected with low doses of spirochetes are protective against intradermal challenge inoculation (8). Although disease undergoes immune-mediated resolution in mice, this process does not correlate with presence of OspA antibody (8, 9). Thus, if OspA immunity following low-dose infection (which parallels natural infection) is not required for protective immunity or disease resolution, it should theoretically not have an influence on infection with host-adapted spirochetes, which apparently do not express significant amounts of OspA. The results of the present study support this position, as mice actively or passively immunized against OspA resisted syringe-borne infection but were fully susceptible to infection by transplantation of tissues which contained host-adapted spirochetes. OspA-immune recipient mice developed disseminated infections, with full inflammatory manifestations in their joints and hearts, which indicate that OspA immunity did not impede the spirochete or disease evolution in any way. It could be argued that the trauma of transplantation or the transplanted tissue itself differed from syringe inoculation sufficiently to protect spirochetes from the host immune response in some way, but our data show that transplantation of normal skin at the site of syringe inoculation did not influence resistance of OspA-immune mice to syringe-borne infection.

We have shown previously that OspA vaccination of mice with existing infections resulted in acceleration of arthritis regression, but the effect was not profound and vaccination did not influence the infection status of the mice (21). In light of our current observations, this OspA immune effect could be explained simply by the nonspecific B-cell mitogenicity of this molecule (16, 34, 42, 53). Hyperimmunization with OspA may nonspecifically stimulate the production of other disease-modulating immune responses, as nonlipidated recombinant OspA-GT, which was used in the study in which mice with existing infections were immunized (21), has been shown to stimulate nonspecific antibody production in splenocytes from uninfected, nonimmune mice (16).

The dose-related immunological response of mice clearly emphasizes a small number of immunodominant proteins, including 22-, 39-, and 41-kDa proteins that are associated with active infection. Sera reactive to these native *B. burgdorferi* proteins also reacted to recombinant OspC (22 kDa), P39 (39 kDa), and flagellin (41 kDa), respectively, consistent with the immungenicities of these proteins following inoculation by low-dose syringe, infected tick, or transplant. Others have also demonstrated reactivity to recombinant P39 of mouse sera obtained from syringe- or tick-infected mice (26, 29). Still other immunogenic proteins may also be present, as immunoblots represent only *B. burgdorferi* antigens that are expressed in vitro. Indeed, recent reports have identified at least two different novel proteins expressed by *B. burgdorferi* in vivo but not in vitro (14, 49).

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