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Oral Vaccination with an Attenuated *Salmonella typhimurium* Strain Expressing *Borrelia burgdorferi* OspA Prevents Murine Lyme Borreliosis

MICHAEL DUNNE,[1,2,3]* BASEL K. AL-RAMADI,[1] STEPHEN W. BARTHOLD,[4] RICHARD A. FLAVELL,[1,4] AND EROL FIKRIG[5]

Sections of Infectious Diseases and Pulmonary Medicine[2] and Section of Rheumatology,[5] Department of Internal Medicine, Section of Immunobiology,[1] and Howard Hughes Medical Institute,[3] School of Medicine, and Section of Comparative Medicine,[4] Yale University, New Haven, Connecticut 06510

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***Borrelia burgdorferi* is the causative agent of Lyme disease. In the mouse model, protection is correlated with the development of antibodies to a major outer surface protein, OspA. In this study, we expressed OspA in an attenuated strain of *Salmonella typhimurium* and tested the efficacy of the transformed strain in protecting against disease. We show that mice inoculated by gavage developed high titers of anti-OspA antibodies and were protected against an intradermal challenge with the spirochete.**

Lyme disease is a consequence of tick-borne infection with the spirochete *Borrelia burgdorferi* (2, 13, 15). Previous work has identified a major outer surface protein, OspA, as the most likely candidate antigen for a Lyme disease vaccine. Mice actively immunized with OspA or passively immunized with polyclonal or monoclonal antibodies to OspA were protected from infection by syringe-injected organisms or from *B. burgdorferi* infection transmitted by a tick bite (8, 10, 22). Furthermore, *B. burgdorferi* were eliminated from ticks that fed on OspA-immunized mice, demonstrating an additional benefit from vaccination, namely, destruction of the pathogen within its vector (11).

We have previously shown that oral administration of *Escherichia coli* expressing OspA protected mice against disease (8, 9). In the present study, we utilized an *aroA* mutant strain of *Salmonella typhimurium* as a carrier for delivering OspA. The use of such mutants as carriers has several advantages. First, they carry a defined, essentially nonrevertant genetic mutation that renders them unable to synthesize essential aromatic compounds and, thus, capable of only limited growth in vivo (6, 12). Second, they have been shown to be safe and efficacious in inducing protective immunity in the mouse model of typhoid fever (12, 14, 17, 19). Similarly attenuated strains of *Salmonella typhi* have also been used successfully in humans to elicit anti-*Salmonella* immune responses (25). Finally, attenuated *Salmonella* strains have been used as carriers of several foreign bacterial, viral, and parasitic antigens and were shown to be effective in inducing appropriate humoral and/or cellular immune responses (1, 7, 18, 20, 21, 23, 27); in some of these cases, protective immunity was also demonstrated (1, 20, 21, 27). In this report, we demonstrate that oral administration of OspA-expressing *S. typhimurium* elicited a protective immune response in inbred mice. These results represent an important step towards an effective oral vaccine for Lyme disease.

For transformation, we used plasmid p197-OspA-N40, whose construction and use have been previously described (8, 9). This plasmid codes for the full-length OspA-N40 protein under the control of the phage lambda p_L promoter and the *c*I857 thermolabile repressor (the latter is active at 30°C and inactive at 42°C) and also contains a tetracycline resistance gene for selection (8). This plasmid was electroporated into *S. typhimurium* SL3235 (kindly provided by Toby Eisenstein, Temple University, Philadelphia, Pa.), a smooth avirulent derivative with a mutation in the *aroA* gene, which has been described previously (3, 12). Transformed colonies were selected on Luria-Bertani agar plates containing 10 μg of tetracycline per ml.

Expression of OspA protein was analyzed by Western blot (immunoblot). After an overnight incubation at 37°C on Luria-Bertani agar, *S. typhimurium* OspA-N40 transformants were grown in tetracycline-containing brain heart infusion broth for 4 h at 30°C. In order to induce the expression of recombinant OspA-N40, some aliquots were incubated at 42°C for a further 2 h. Protein extracts, prepared as described elsewhere (24), were boiled for 5 min and run on 12.5% polyacrylamide–sodium dodecyl sulfate (SDS) gels under nonreducing conditions. The proteins were transferred to a nitrocellulose filter in Tris–glycine–20% methanol buffer and blocked for 2 h at 25°C in phosphate-buffered saline (PBS) containing 5% nonfat dried milk and 0.02% sodium azide. The filter was incubated for 1 h at 25°C with a 1/100 dilution of C3.78 monoclonal antibody (which is specific for the C terminus region of OspA [24]), washed three times in PBS containing 0.05% Nonidet P-40, and then similarly incubated with horseradish peroxidase-conjugated goat anti-mouse immunoglobulin G (IgG) Fc-specific antibody (Accurate, Westbury, N.Y.). At the end of the second incubation, the filter was washed three times and color was developed with 4-chloro-α-naphthol as a substrate. The image was then digitized with a Howtech scanner and Adobe Photoshop software. The results of the immunoblot analysis (Fig. 1) show that, when transformed SL3235 organisms were grown at 30°C and shifted to 42°C for the final 2 h, a C3.78-reactive band of appropriate size (~31 kDa) was observed (lane 1). This band, however, was absent when transformants were grown at 30°C only (lane 2) or when nontransformed SL3235 organisms were examined (lane 3).

We then examined whether SL3235 OspA-N40 transformants expressed OspA on the cell surface by flow cytometry. For this, the bacteria were grown as outlined above and washed twice in staining buffer (SB) (PBS–1% fetal calf se-

* Corresponding author. Present address: Pfizer Central Research, Eastern Point Rd., Groton, CT 06340. Phone: (203) 441-3739. Fax: (203) 441-5702.

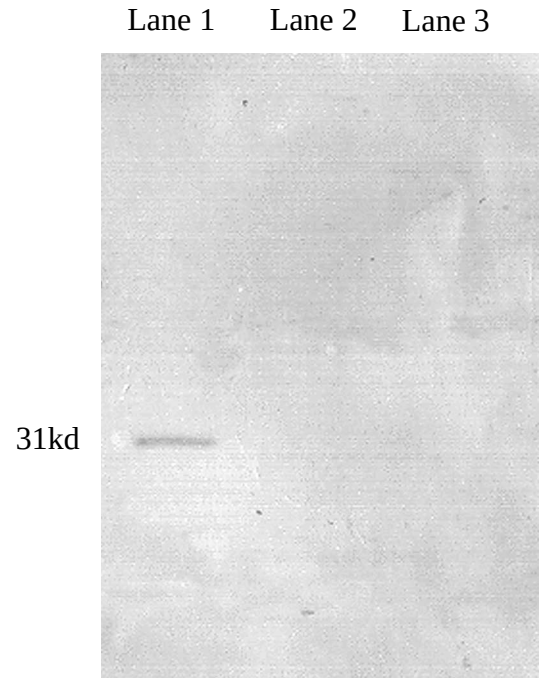

FIG. 1. Western blot analysis of bacterial extracts derived from untransformed *S. typhimurium* SL3235 (lane 3) or SL3235 OspA transformants grown at 30°C only (lane 2) or at 30°C and then for 2 h at 42°C (lane 1). The material was run on an SDS–12.5% polyacrylamide gel, transferred to nitrocellulose paper, and probed with the C-terminus-specific monoclonal antibody. The C3.78 band at 31 kDa represents OspA.

rum–0.1% sodium azide). Three different OspA-specific monoclonal antibodies were used: C3.78 (IgG3) and H3TS (IgG2a), both of which bind to the C terminus of OspA, and 8C4BC (IgG2b), which is specific to the OspA N terminus (24). Those primary monoclonal antibodies were diluted 1/100 in SB, added to bacterial pellets in Eppendorf tubes, and mixed thoroughly, and the samples were incubated for 30 min on ice. Cells were then washed three times and incubated with fluorescein isothiocyanate-conjugated goat anti-mouse IgG (HyClone, Logan, Utah) secondary antibody for 30 min on ice. Cells were again extensively washed in SB, resuspended in 1 ml of SB, and transferred to 12-mm test tubes. Analysis was performed in a FACS Star Plus (Becton Dickinson, Mountain View, Calif.), and the data were analyzed with LYSYS II software. As shown in Fig. 2, a significant percentage of OspA-N40 transformants were labeled with the two C-terminus-specific antibodies C3.78 and H3TS. In contrast, no staining was observed with 8C4BC antibody directed against the N terminus of OspA.

We next evaluated the ability of SL3235 OspA-N40 transformants to induce anti-OspA antibodies. (All animal studies were approved by the University Institutional Animal Care and Use Committee.) C3H/HeJ mice (Jackson Laboratory, Bar Harbor, Maine), at 8 weeks of age, were inoculated by gavage with 10^8 organisms of either the SL3235 OspA transformant or nontransformed SL3235. Bacteria were grown for 4 h at 30°C, shifted to 42°C for another 2 h (as described above), adjusted to the desired concentration, and administered in 200 μl of PBS. Mice were boosted by a total of five weekly inoculations, and sera were collected by retro-orbital sinus puncture. Immu-

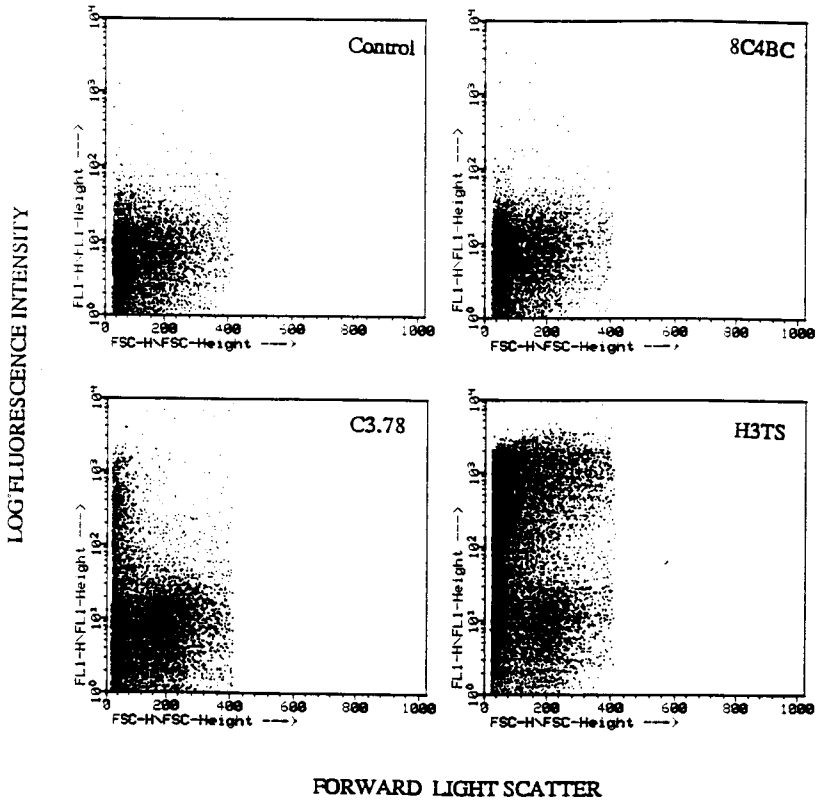

FIG. 2. Flow-cytometric analysis of surface expression of OspA in SL3235 OspA transformants. Bacteria were stained with secondary antibody only (control), N-terminus-specific monoclonal antibody 8C4BC, or C-terminus-specific monoclonal antibody C3.78 or H3TS. A total of 15,000 bacteria were analyzed per sample. The results are presented as dot blots depicting logarithmic fluorescence intensity as a function of forward light scatter (indicative of cell size).

TABLE 1. OspA antibody titers in mice orally administered *S. typhimurium* expressing OspA[a]

Serum dilution	No. of experimental mice with a detectable antibody response ($n = 10$)	P^b
1:100	9	<0.001
1:500	8	0.001
1:5,000	4	0.069
1:50,000	0	NS

[a] Sera obtained from mice after five doses of SL3235 OspA-N40 transformants (experimental group) or parent SL3235 (control group), respectively, were analyzed by immunoblots.
[b] With respect to the results for the eight control mice, none of which developed a detectable antibody response. NS, not significant.

TABLE 2. Protection of mice immunized with SL3235 OspA-N40 transformants against *B. burgdorferi* infection[a]

Immunization	No. of mice with[b]:				
	Positive culture[c]			Carditis	Infection[d]
	Blood	Bladder	Any site		
SL3235	4/6	7/7	7/8	7/8	7/8
SL3235 OspA-N40	0/7	2/10	2/10	2/10	2/10
P	0.021	0.002	0.008	0.008	0.008

[a] Animals with either a positive culture or disease were considered to be infected with *B. burgdorferi*.
[b] The denominators vary because of contamination of some cultures, which were subsequently excluded from analysis.
[c] None of the five spleen samples for either group were positive.
[d] Any site.

noblotting was performed on nitrocellulose paper to which 1 μl of a 10-μl/ml solution of OspA had been added and then blocked with reconstituted evaporated milk. The nitrocellulose paper was then incubated with sera from the vaccinated animals, washed, and developed with horseradish peroxidase-goat anti-mouse IgG and a substrate. The data shown in Table 1 summarize the results obtained after the fifth boost. As can be seen, 9 of 10 mice immunized with SL3235 OspA-N40 organisms developed an antibody response to OspA detectable at a dilution of 1:100 or greater by immunoblot (Table 1). Of the sera from those nine mice that showed strong anti-OspA antibody responses, sera from two mice, chosen at random, were further tested and found to have titers of greater than 1:100 against a C terminus fragment of OspA, designated fragment 8 (amino acids 133 to 273), known to contain the protective epitopes (data not shown) (4, 24). Importantly, all eight mice immunized with the parent SL3235 strain failed to develop any detectable OspA-specific antibodies.

In order to determine whether oral immunization with SL3235 OspA-N40 transformants could protect against virulent infection with *B. burgdorferi*, the above immunized groups of mice were challenged 7 weeks after the last boost by an intradermal injection of 1.5×10^3 organisms of *B. burgdorferi* N40, a strain with proven pathogenicity in mice (8). The conditions used to grow *B. burgdorferi* have been detailed previously (8). Two weeks after challenge, the animals were sacrificed and examined for infection as previously described (8). Cultures of blood, bladder, and spleen were incubated in Barbour-Stoenner-Kelly medium for 2 weeks and then examined by dark-field microscopy. The presence of any organisms in 20 high-power fields constituted a positive culture, which was considered indicative of infection. In addition, histopathologic examinations were performed on hearts collected from immunized mice. The slides were examined in a blinded fashion for evidence of inflammation, as previously described (11). Statistical analysis was performed by the use of a one-sided Fisher's exact test.

Results of the above analysis revealed that 8 of 10 mice immunized with SL3235 OspA-N40 had negative cultures from all sites (Table 2). The two animals with positive cultures had organisms isolated only from the urinary bladder wall (Table 2). Histopathologic examination of heart tissue revealed evidence of carditis in 2 of 10 immunized mice, the same two mice which had positive bladder cultures (Table 2). These results are in sharp contrast to those observed for mice immunized with the parent SL3235 strain: of the eight mice immunized with the parent strain, seven showed positive cultures and developed carditis, a finding constituting clear evidence of active infection.

These data demonstrate that oral immunization with attenuated *S. typhimurium* expressing OspA resulted in the induction of a good antibody response to OspA and protected against challenge with virulent *B. burgdorferi*. Fluorescence-activated cell sorter (FACS) analysis of SL3235 OspA-N40 transformants showed that OspA is expressed on the cell surface. The finding that the surface expression of OspA was recognized mainly by antibodies directed against its C terminus suggests that the orientation of OspA in salmonella is such that the C terminus is exposed and the N terminus is buried within the cell membrane. It is likely that the OspA is localized in a similar fashion on the *B. burgdorferi* surface since we have previously shown that only anti-C-terminus antibodies were protective in passive immunization studies and surface-exposed epitopes are essential for binding to antibodies that kill the spirochete (24).

The results of the protection experiment showed that 8 of 10 immunized mice were protected from disease. Failure of vaccination in two mice could be the result of low inocula or due to problems with in vivo plasmid instability.

This vaccine protected mice against a spirochetal challenge well in excess of the estimated exposure that might occur in nature (26). As vaccines derived from transformed salmonella have been shown to generate appropriate humoral and/or cellular immune responses (5, 18, 20, 21, 23, 27), are safe in humans (16, 25), and offer clear technical and economical advantages over those requiring protein purification, an attenuated salmonella vaccine expressing OspA would likely prove to be a useful strategy for immunization against Lyme disease.

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