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Biological Activities of Native and Recombinant *Borrelia burgdorferi* Outer Surface Protein A: Dependence on Lipid Modification

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***Borrelia burgdorferi* lipoproteins are 50- to 500-fold more active as cytokine inducers and B-cell mitogens than *Escherichia coli* lipoproteins and synthetic peptides containing the tripalmitoyl-*S*-glyceryl-cysteine moiety. To investigate the source of this unique potency, we compared native OspA from *B. burgdorferi* with recombinant lipidated OspA produced in *E. coli*. As little as 10 ng of either protein per ml stimulated B-cell proliferation and production of cytokines and nitric oxide by macrophages. The two proteins induced comparable antibody responses in mice. Nonlipidated OspA made in *E. coli* had no stimulatory activity. Thus, lipid modification is essential both in vivo and in vitro for the immunological properties of OspA. The lipid moiety appears equally active whether produced in *B. burgdorferi* or in *E. coli*.**

OspA, the major outer surface lipoprotein of the Lyme disease pathogen, *Borrelia burgdorferi*, provides protective immunity in animal models (12, 24). OspA contains a posttranslationally added tripalmitoyl-*S*-glyceryl-cysteine (Pam_3Cys) lipid moiety at its N terminus (5). The Pam_3Cys lipid moiety is essential for induction of the immune response in mice (11), even though antibodies are directed principally against the protein part of the molecule (11, 12, 24). Native, lipidated OspA purified from *B. burgdorferi* in both mice and humans is a B-cell mitogen and stimulates production of inflammatory cytokines by macrophages, monocytes, and endothelial cells (7, 17, 22, 25, 26). Native OspA is active at molar concentrations 50- to 500-fold lower than those reported for the Braun lipoprotein of *Escherichia coli* or for a variety of lipopeptides containing Pam_3Cys (3, 13, 14, 19). These findings suggested that the extremely high potency of the *B. burgdorferi* lipoproteins could be the result of unique modifications made by the spirochete.

In order to test this hypothesis, we compared the biological properties of native OspA purified from *B. burgdorferi*, recombinant lipidated OspA purified from *E. coli*, and recombinant nonlipidated OspA purified from *E. coli*. Native OspA was purified by affinity chromatography with monoclonal antibody H5332 (1) from an *n*-butanol extract of the N40 clone of *B. burgdorferi*, as described before (17), and stored in 10 mM Tris (pH 7.4)–5 mM NaCl–2 mM EDTA. Recombinant lipidated OspA was purified from the Triton X-114 detergent phase of a lysate of *E. coli* expressing the full-length B31 *ospA* gene, including the 16-amino-acid lipoprotein signal sequence (11). The recombinant lipoprotein was stored in 50 mM Tris (pH 7.5)–10 mM NaCl–2 mM EDTA–0.3% Triton X-100. The recombinant nonlipidated OspA (a gift of John Dunn, Brookhaven National Laboratories) was prepared as described previously (10) from *E. coli* expressing a B31 *ospA* gene truncated for the signal peptide. Nonlipidated OspA was stored in 10 mM sodium phosphate (pH 6.0)–50 mM NaCl. Protein concentrations were determined by the Lowry assay (16). Contamination with bacterial endotoxin in all three preparations was less than 0.3 endotoxin units (EU) per 500 ng of OspA, the minimal amount detectable with the *Limulus* amebocyte lysate assay (Associates of Cape Cod, Inc.).

The ability of OspA to induce lymphocyte proliferation was assessed by culture of 2×10^5 splenocytes from naive C3H/HeJ mice per well for 3 days in 0.2 ml of RPMI containing 1% (vol/vol) of the serum replacement Nutridoma SR (Boehringer Mannheim), 0.05 mM 2-mercaptoethanol, 2 mM L-glutamine, 0.01 mg of gentamicin sulfate per ml, and 10 μg of polymyxin B per ml (LM). A total of 1 μCi of [^{3}H]thymidine (Amersham

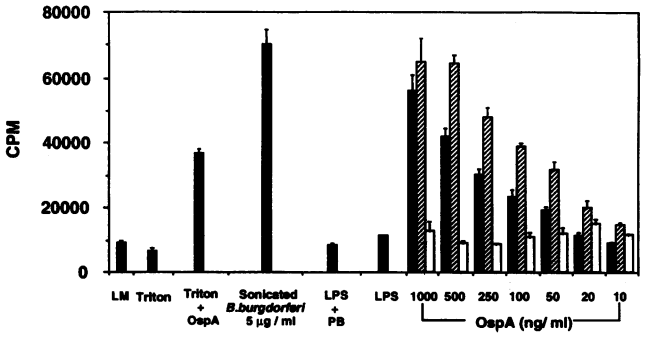

FIG. 1. Mitogenic activity of OspA purified from *B. burgdorferi* or *E. coli*. Lipidated OspA was purified from *B. burgdorferi* (filled bars) or from a recombinant expressing *E. coli* (hatched bars). Nonlipidated OspA was purified from *E. coli* (open bars). OspA was added at the indicated concentration to splenocytes from naive C3H/HeJ mice, and proliferation was measured after 72 h. The sample labeled LM received medium alone, the two samples labeled Triton received the same final concentration of Triton X-100 as the higher recombinant OspA sample, and the sample labeled Triton + OspA contained Triton X-114 and 500 ng of native OspA per ml. Sonicated *B. burgdorferi* was a positive control, and lipopolysaccharide (LPS) samples received 100 ng of bacterial endotoxin (Sigma) per ml. Values represent the means and standard deviations of triplicate samples. PB, polymyxin B.

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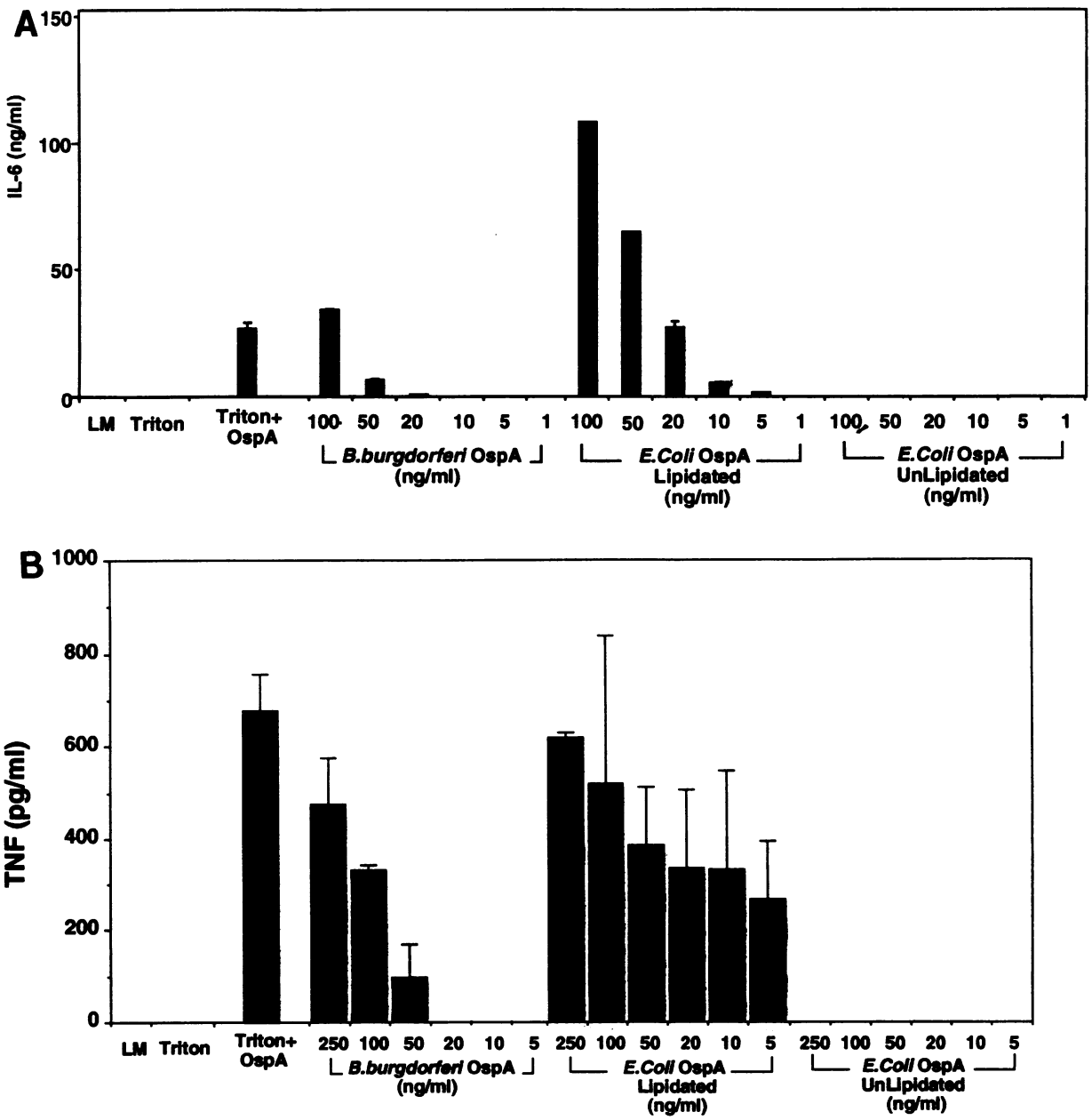

FIG. 2. Cytokine production by macrophages incubated with OspA from *B. burgdorferi* or *E. coli*. Bone marrow-derived macrophages, 3×10^5, from C3H/HeJ mice were incubated with the indicated concentration and source of OspA. Supernatants were assayed for interleukin-6 (A) and tumor necrosis factor alpha (B) at 48 h. Abbreviations and labeling of samples are as given in the legend to Fig. 1. Values represent the means and standard deviations of triplicate samples.

Corp.) was added for the last 20 h of culture, and isotope incorporation into DNA was determined from samples recovered with a PHD cell harvester (Cambridge Technologies), as described before (17). We have previously demonstrated that this assay measures proliferation of B lymphocytes in splenocyte preparations from naive mice (17). Similar dose-response curves were obtained with native OspA purified from *B. burgdorferi* and recombinant lipidated OspA (Fig. 1). The nonlipidated recombinant did not stimulate proliferation above background. The somewhat higher values seen with the recombinant lipidated OspA relative to the native protein may reflect the presence of slightly inhibitory levels of residual detergent in the native OspA (17, 25). The activity of the two lipoproteins is not due to the presence of residual endotoxin, as evidenced by the facts that proliferation was not inhibited by the endotoxin inhibitor polymyxin B (21) and it was seen in lymphocytes from endotoxin-resistant C3H/HeJ mice. Similar results were obtained with splenocytes from BALB/c mice (data not shown).

Cytokine and nitric oxide (NO) production was assessed in cultures of bone marrow-derived macrophages from C3H/HeJ mice incubated with sonicated *B. burgdorferi* or with the three preparations of OspA. Macrophages were prepared as described before (18) and cultured in LM for 48 h with the

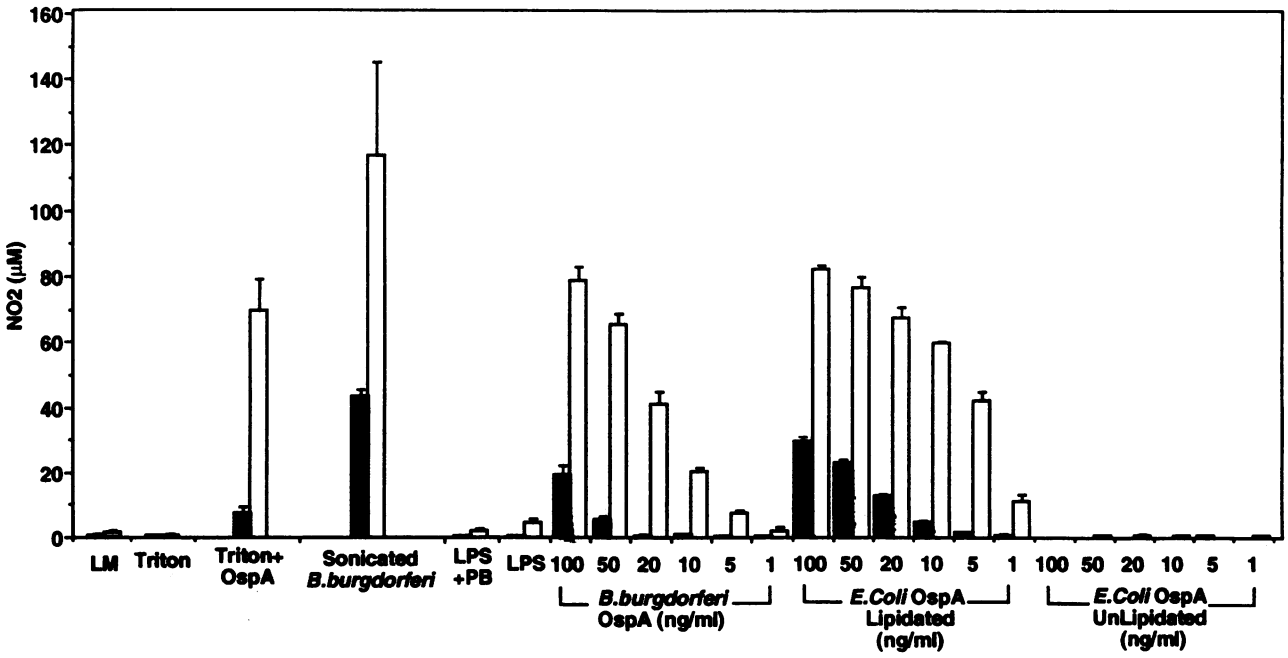

FIG. 3. Cytokine-inducible NO production by bone marrow-derived macrophages incubated with OspA. Macrophages were incubated with the indicated source and concentration of OspA for 24 h in the absence (filled bars) or presence (open bars) of 1 U of gamma interferon per ml. Supernatants were assessed for nitrite presence as an indication of NO production. Abbreviations and labeling of samples are as given in the legend to Fig. 1. Values represent the means and standard deviations of triplicate samples.

indicated stimuli. In addition, both lipoprotein preparations were very potent stimulators of macrophage function as determined by detection of interleukin-6 and tumor necrosis factor alpha in the culture supernatants by antibody capture enzyme-linked immunosorbent assay (ELISA) (Fig. 2) (17). Both lipidated forms of OspA were potent stimulators of NO production, as measured by the Greiss reaction (Fig. 3) (8). NO is a potent antimicrobial agent, and its induction could be important in controlling bacterial growth (9). The presence of gamma interferon, which up-regulates the production of the cytokine-inducible NO synthase (9), caused NO production by macrophages incubated with as little as 5 ng of recombinant OspA and 10 ng of OspA from *B. burgdorferi* per ml. None of the macrophage responses were stimulated by the nonlipidated OspA, indicating that the lipid modification was crucial for the biological properties. It is likely that interaction of the lipid moiety with a specific receptor on macrophages and B lymphocytes mediates this response, although such a molecule has not been characterized. The similar dose-response curves for OspA produced by *E. coli* and *B. burgdorferi* argue that *B. burgdorferi* lipoproteins are not inherently more active than *E. coli* lipoproteins.

B-cell mitogen activity was identified with the Braun lipoprotein 20 years ago; however, in those early experiments a much greater amount of material was required for stimulation (19). Experiments with the purified Braun protein have not been repeated recently, nor has cytokine analysis been performed. It is possible that the higher concentration of the Braun lipoprotein required for activity reflects changes in the mitogen assay or differences in the degree of aggregation or solubility inherent in the very different purification protocol used for the Braun lipoprotein (19). The lower activity on a molar basis of synthetic lipopeptides may reflect solubility differences or perhaps a chemical heterogeneity in the synthetic lipid moiety (3, 13, 14). The findings presented in Fig. 1 to 3 do not address the fatty acid compositions of the lipid modification attached by the two bacterial strains. However, they demonstrate that there is not a greater biological activity attributable to the presence of unique lipid modification of *B. burgdorferi* synthesized lipoproteins. Electrospray mass spectroscopy of the recombinant lipidated OspA revealed three major molecular species (4). One was consistent with the fully lipidated Pam_3Cys structure, the second was consistent with a lipoprotein lacking one of the fatty acid residues, and the third species was not identified (6).

The ability of recombinant OspA to induce an antibody response in mice was previously shown to depend on the presence of a lipid modification consistent with the Pam_3Cys structure of the amino-terminal modification of the Braun lipoprotein (11), although antibody response to OspA is principally to the polypeptide portion of the molecule (11, 12, 24). The immunogenicities of native and recombinant lipidated OspA were compared (Fig. 4). C3H/HeN mice were immunized subcutaneously on days 0 and 21 with 0.2 or 1 μg of purified OspA without added adjuvant (11). Serum taken on day 28 was analyzed by ELISA, using recombinant OspA lipoprotein as the plate antigen. Consistent with their similar in vitro stimulatory activities, the two lipoproteins induced similar immunoglobulin G responses, supporting the notion that there is nothing unique about the lipid modification produced in *B. burgdorferi*. The Pam_3Cys lipid moiety is a potent adjuvant, since immunization with as little as 0.4 μg of OspA lipoprotein in the absence of added adjuvant protected mice completely against spirochete challenge, while OspA lacking the lipid moiety induced no detectable immune response (11). Pam_3Cys was in itself a sufficiently strong adjuvant that adsorption of OspA lipoprotein to alum did not significantly enhance the antibody response, either in mice or in a phase I human trial (11, 15). It is likely that the potent stimulatory properties of the

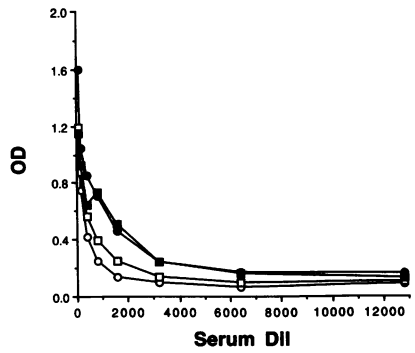

FIG. 4. Immunoglobulin G response to OspA from *B. burgdorferi* and *E. coli*. C3H/HeN mice were injected subcutaneously with purified *B. burgdorferi* OspA (□, 0.2 μg; ■, 1.0 μg) or lipoprotein recombinant *E. coli* OspA (○, 0.2 μg; ●, 1 μg). Mice were given booster injections at day 21, and serum immunoglobulin G levels were assessed at day 28 by recombinant OspA ELISA. Plates were read in an ELISA reader, and results were plotted as optical density (OD) units (*x* axis) versus the reciprocal of the dilution (*y* axis).

Pam_3Cys lipid moiety are, in part, responsible for its adjuvant activities.

These findings indicate that lipidated OspA is a good immunogen and potent biological stimulator in mice. While the lipid modification is required for these activities, no differences are apparent between the lipidation produced in *B. burgdorferi* and that produced in *E. coli*. It is interesting that OspA is active at considerably lower concentrations than have been reported for other bacterial lipoproteins, especially the Braun lipoprotein of *E. coli* (19). Although the reason for this is not clear, it is possible that the purification methods developed for OspA have maintained the protein in a conformational state that is more optimally recognized by lymphocytes. The differences may also reflect the reliance in many recent studies on synthetic lipopeptides which may differ somewhat in their lipid modification from natural lipoproteins (3, 13, 14). The extreme potency of OspA, especially as demonstrated with the cytokine-inducible NO production, could explain how small numbers of spirochetes invading a site could result in intense inflammation (27). Understanding of the parameters determining immune stimulation (23, 28), inflammatory stimulation (2, 6, 20), and anergy to OspA will require full understanding of the structure and presentation of this biologically active molecule.

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