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Borrelia burgdorferi Outer Surface Lipoproteins OspA and OspB Possess B-Cell Mitogenic and Cytokine-Stimulatory Properties

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Sonicated *Borrelia burgdorferi* was previously reported to possess both B-cell mitogenic and interleukin-6 (IL-6) stimulatory activities. In this report, two outer surface lipoproteins, OspA and OspB, were purified from *B. burgdorferi* and assessed for the presence of these functions. OspA was purified from two strains, an OspB-deficient variant of HB19 and N40, while OspB was purified from the N40 strain. All lipoprotein preparations were free of endotoxin contamination, and polymyxin B failed to inhibit responses, indicating that media contamination was not contributing to biological assays. All three preparations were able to stimulate proliferation of mononuclear cells from naive C3H/HeJ and BALB/c mice. Depletion experiments indicated that the responding cells were B lymphocytes and not T lymphocytes. Purified OspA and OspB stimulated immunoglobulin M production by splenocyte cultures from naive mice, a property also previously attributed to sonicated *B. burgdorferi*. OspA and OspB also stimulated the production of IL-6 and tumor necrosis factor alpha by bone marrow-derived macrophages from BALB/c and C3H/HeJ mice. Cytokine production was enhanced by the presence of gamma interferon in the cultures, indicating that the magnitude of responses to these lipoproteins may be modulated by cytokines in the microenvironment of infected tissues. Human endothelial cells produced IL-6 when incubated with OspA and OspB, indicating that non-hematopoietic lineage cells can respond to the lipoproteins. Purified OspA and OspB had approximately equal activity, with responses detected in the range of 10 ng of lipoprotein per ml to 1 μg of lipoprotein per ml. Comparison with published dose responses for lipoproteins purified from *Escherichia coli* indicates that OspA and OspB purified from *B. burgdorferi* are much more potent. The high potency of the *B. burgdorferi* lipoproteins and the ability of the spirochete to invade tissues and persist argue that they could be important in the localized events contributing to the pathology of Lyme disease.

Lyme disease in humans results from infection by the tick-borne spirochete *Borrelia burgdorferi* (10). Many organs and tissues can be involved in late-stage disease, with transient, intermittent arthritis being the most common in untreated individuals (43). Persistent infection by the spirochete is clearly important in pathological developments, as demonstrated by the effect of antibiotics (4, 40, 43). Many symptoms may result from the invasion of tissues by the spirochete or from localized aspects of the host's immune and inflammatory response (4, 40). A major goal of our studies is to understand the contribution of bacterial factors and host defenses to the events leading to arthritis development in *B. burgdorferi*-infected mice.

We reported that *B. burgdorferi* possessed a potent mitogen for murine B cells, capable of stimulating polyclonal B-cell proliferation and immunoglobulin production (39). *B. burgdorferi* also stimulated interleukin-6 (IL-6) production by mononuclear cells. Neither of these activities could be inhibited with polymyxin B, an inhibitor of the lipid A moiety of bacterial endotoxin, whereas all similar activities in a sonicate prepared from *Escherichia coli* could be inhibited by polymyxin B. This suggested that *B. burgdorferi* has unique, potent, cellular stimulatory activities that could be involved in the pathogenesis of infection. de Souza et al. (16) have also identified B-cell mitogen activity in the sonicated *B. burgdorferi*, while Whitmire and Garon (47) have found this activity highly concentrated in the extracellular blebs released from the organism. Several groups have characterized cytokine production by various cell types incubated with *B. burgdorferi*, including reports of IL-6 production by glial cells (20), IL-1 production by human and bovine cells (24, 33), and tumor necrosis factor alpha (TNF-α) production by murine and human cells (15). Furthermore, IL-1 has been identified in synovial fluids of arthritic patients (5, 33), and TNF-α has been found in the sera and synovial fluid of patients (15). Hyperactive B cells have also been found in Lyme disease patients (41). With the murine model for severe arthritis developed by Barthold et al. (3), we demonstrated that both the B-cell mitogen and the cytokine stimulatory activities function in vivo (47). *B. burgdorferi*-infected mice of the severely arthritic C3H/HeJ strain had a 15-fold expansion of B lymphocytes in the peripheral nodes and a 10- to 20-fold elevation in circulating immunoglobulin G (IgG). Profound cytokine stimulation was also found, with serum IL-6 levels up to 10 ng/ml. Since several investigators have reported cytokine production and hyperactivation of B lymphocytes during Lyme arthritis, these findings in the mouse may correlate with events in the human (5, 15, 33, 40, 47). To further study the cellular responses to *B. burgdorferi* and potential roles in pathogenesis, we wished to identify and purify the biologically active molecules. This study reports that purified OspA and OspB possess both the B-cell mitogen and cytokine stimulatory properties.

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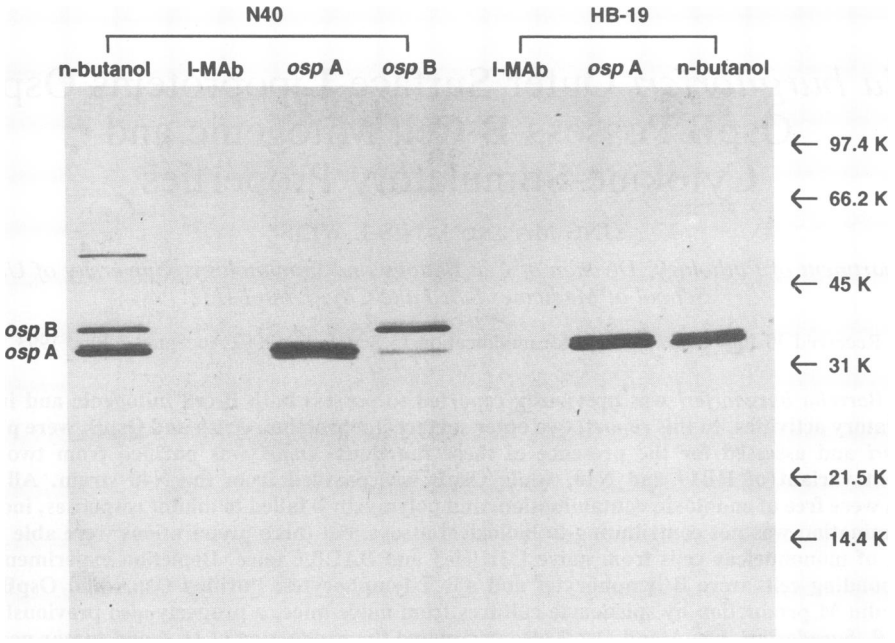


FIG. 1. Purification of OspA and OspB from *B. burgdorferi*. A total of 500 ng of purified OspA or OspB or the *n*-butanol extract was analyzed by SDS–12% PAGE and detected by silver staining. An equivalent volume of eluate from an irrelevant monoclonal antibody column was loaded in the I-MAb lane. OspA was purified from the OspB-negative strain HB-19 and from the cloned N40. OspB was purified from the cloned N40 strain. The dye front was retained on the gel, with no evidence for low-molecular-weight contaminants. The position of migration of molecular mass markers is shown on the right of the gel, and the positions of OspA and OspB are indicated on the left.

MATERIALS AND METHODS

Mice. C3H/HeJ ($H\text{-}2^k$) and BALB/c ($H\text{-}2^d$) mice were obtained from Jackson Laboratories. Mice were housed in the Animal Resource Center of the University of Utah Medical Center.

Bacteria. The N40 isolate of *B. burgdorferi* was provided by Stephen Barthold, Yale University, at passage 3 from an infected mouse (3). This strain contained a mixture of bacteria expressing full-length OspB of 34,000 M_r and truncated OspB of 20,000 M_r. A clone expressing only the full-length form of OspB was obtained by plating on solid media (37) and was identified by Western blot (immunoblot) analysis of spirochete proteins in cultures derived from individual colonies. An OspB-negative variant of *B. burgdorferi* HB19 was provided by Alan Barbour, University of Texas (38). This variant was selected by growth in the presence of monoclonal antibody to OspB, H614 (9), passaged 10 times without antibody, and cloned (37a). No OspB was detected by silver staining of a lipoprotein-enriched *n*-butanol extract of this strain (Fig. 1), nor was OspB detected by Western blotting with H4610, a monoclonal antibody shown to bind to many of the naturally occurring truncated OspB proteins (37). Cultures were maintained as 0.5-ml frozen stocks at −70°C. Fresh aliquots were seeded in 15 ml of Barbour Stoenner Kelly II medium (BSK II [1]) and cultured at 32°C.

Monoclonal antibodies. Hybridoma clones GK1.5 (anti-CD4 [17]), 53-6.7 (anti-CD8 [26]), and 30-H12 (anti-Thy-1.2 [26]) were obtained from the American Type Culture Collection. Hybridoma clones producing monoclonal antibodies H5332 (anti-OspA [2]) and H614 (anti-OspB [9]) were provided by Alan Barbour. The hybridomas were grown in RPMI containing a 1% volume of the serum replacement Nutridoma SR (Boehringer Mannheim) and recovered by precipitation of the culture supernatant with 50% saturated ammonium sulfate. The low protein content of Nutridoma-containing medium allowed the recovery of highly enriched immunoglobulin, which was used directly for preparation of affinity columns. Monoclonal antibody H4610 (anti-OspB [37]) was provided by Tom Schwan, Rocky Mountain Laboratories.

Purification of OspA and OspB. OspA was purified from 600-ml cultures of the OspB-negative variant of *B. burgdorferi* HB19 (38). Spirochetes were collected by centrifugation at 3,200 × *g* for 30 min. The pellet was washed twice and resuspended in 7 ml of phosphate-buffered saline (PBS), pH 7.4, containing 5 mM $MgCl_2$ and sonicated twice for 45 s (each) on ice with a cell disruptor on level 6 (Heat Systems-Ultrasonics, Inc., model W-220f). The membrane-enriched fraction of *B. burgdorferi* was collected by centrifugation at 100,000 × *g* for 90 min. The pelleted material was suspended in 10 ml of PBS and sonicated for 15 s at setting 6 to aid resuspension. The suspension was mixed with 8 ml of *n*-butanol and incubated with gentle rocking for 1 h at 4°C (19). The aqueous phase was recovered by centrifugation at 27,000 × *g* for 90 min at 4°C and dialyzed against a solution of 10 mM Tris (pH 7.4), 5 mM NaCl, 2 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, 1 μM pepstatin, and 1 μM leupeptin (chromatography starting buffer). The starting material was made 30 mM with *n*-octyl-β-glucoside (Boehringer Mannheim) and applied sequentially to a series of precolumns and affinity columns. All columns were prepared by coupling 3 ml of CNBr-activated Sepharose with 9 mg of purified protein. The first column was a bovine serum albumin (BSA)-Sepharose column. The second column consisted of Sepharose coupled to an unrelated IgG2A monoclonal antibody, HB-5, which binds human complement receptor 2 (45) and was shown not to react with *B. burgdor-*

feri proteins by Western blot analysis. Material not bound by either of the precolumns was then applied to a monoclonal anti-OspA column. The column was washed with detergent-containing buffer, and the bound material was eluted with 0.2 M acetic acid (pH 2.5) containing 5 mM NaCl. The eluate was neutralized with 1 M Trizma base and dialyzed against three changes of starting buffer to remove the *n*-octyl-β-glucoside. The OspA remained soluble for months in this low-salt buffer, as reported by others (35); however, precipitates did form if dialyzed into buffer containing 0.15 M NaCl. Column fractions were stored at 4°C, and protein concentration was determined by Folin assay (27). The yield of purified OspA from a 600-ml culture was 50 μg. The OspA was homogeneous, as determined by silver stain (Bio-Rad) analysis of a 12% sodium dodecyl sulfate (SDS)–polyacrylamide gel (25), with no evidence for contamination with low-molecular-weight species (Fig. 1).

OspB and OspA were purified from the full-length OspB-expressing clone of N40. The procedure was identical to that used to purify OspA from the OspB-deficient variant, with the detergent containing starting material applied sequentially to BSA-Sepharose, HB-5 Sepharose, and anti-OspA (H5332) or anti-OspB (H614) Sepharose. A small proportion of OspA and OspB were tightly associated, as demonstrated by the finding that material eluted from the first of the two monoclonal antibody affinity columns for the lipoproteins was contaminated with about 20% of the other lipoprotein, determined by analysis of a silver-stained gel with a Bio-Rad densitometer. This contamination was somewhat reduced in the material eluted from the second antibody column. Therefore, the OspA and OspB purified from N40, shown in Fig. 1 and used in biological studies, were the eluates from the second anti-Osp column of the sequential purification. The yield of OspB from the N40 clone was 370 ng, and that of OspA was 50 ng. The greater yield of OspB probably represents a greater binding efficiency of the monoclonal antibody H614 for OspB, as there actually appears to be more OspA than OspB in the silver-stained gel of material in the *n*-butanol extract (Fig. 1). Several other detergents were tested for the ability to more completely separate OspA and OspB, including Triton X-100, Triton X-114, SDS, Nonidet P-40, and 3-[(3-cholamidopropyl)-dimethyl-ammonio]-1-propanesulfonate (CHAPS), but none provided an advantage over the dialyzable *n*-octyl-β-glucoside.

Proteinase K digestion. Proteinase K digestion was performed on purified OspB or sonicated *B. burgdorferi* suspended in 50 mM Tris, pH 8.0, with 1 mM $CaCl_2$ by adding 1 μg of proteinase K per 2.5 μg of protein and incubating for 6 h at 37°C. The reaction was stopped by the addition of 0.5 mM phenylmethylsulfonyl fluoride. Controls were incubated in buffer without proteinase K. Silver stain analysis of 1 μg of the digests and controls run on a SDS–12% polyacrylamide gel revealed the digestion to be complete (data not shown).

Endotoxin analysis. The level of endotoxin contamination in purified OspA and OspB and in the tissue culture medium was assessed with the *Limulus* amebocyte lysate assay. Tests were performed according to the manufacturer's (Associates of Cape Cod, Inc.) directions. The assay detected as little as 0.03 endotoxin units (EU) per ml of the *E. coli* endotoxin 0113:H10, with a conversion of 5 EU per ng. All preparations of OspA and OspB contained less than 0.03 EU per 500-ng sample. Water used to prepare media and chromatography buffers had been filtered through a Barnstead Nanopure II system equipped with the ULTRAfilter accessory kit for the removal of pyrogens and was endotoxin free. All glassware used in media and buffer preparation had been baked to remove endotoxin, and equipment that could not be baked was washed with the detergent E-Toxa-Clean (Sigma). Lymphocyte medium containing 1% Nutridoma, antibiotics, glutamine, and 2-mercaptoethanol was found to contain 2 EU/ml (equal to 0.4 ng of the *E. coli* endotoxin standard per ml). Endotoxin concentrations 1,000 to 10,000 times higher were inhibited by the 10 μg of polymyxin B per ml added to the tissue culture. Assays run in the presence and absence of polymyxin B gave similar results, further indicating that the very low level of endotoxin in the culture media was not affecting results or increasing background values.

The commercial endotoxin used in the mitogen and cytokine stimulatory assays was from *Salmonella typhosa* (Sigma) and was used at 1 μg/ml for B-cell mitogen assays and 100 ng/ml for macrophage cytokine stimulatory assays. Cells from C3H/HeJ mice responded weakly or not at all to the commercial lipopolysaccharide (LPS) while cells from BALB/c mice always responded intensely.

Depletion of B lymphocytes. Single-cell suspensions of splenocytes were prepared at a concentration of 2×10^7 per ml in PBS containing 5% fetal calf serum. Of this suspension, 3 ml was layered on bacterial petri dishes coated with goat anti-mouse IgG, IgM, and IgA (Zymed) (12). The dishes were incubated for 70 min at 4°C, and the unbound cells were recovered. The depletion step was repeated a second time for optimal depletion of B lymphocytes. Flow cytometry analysis indicated that before depletion, 45% of cells stained positively with fluorescein isothiocyanate-conjugated goat anti-mouse IgM (Bethesda Research Laboratories), while after depletion 1% of remaining cells were positive.

Depletion of T lymphocytes. Efficient depletion of T cells was achieved by a combination of in vivo and in vitro steps (12). C3H/HeJ or BALB/c mice received an intraperitoneal injection of 100 μg of rat monoclonal antibodies to CD4 and CD8. The animals were sacrificed 5 days later, and single-cell suspensions of splenocytes were prepared. Cells were incubated with a saturating concentration of rat anti-Thy-1.2 for 30 min on ice. Unbound antibody was removed by centrifugation, and the cells were suspended in 10% Low-Tox-M rabbit complement (Cedarlane Laboratories) and incubated at 37°C for 30 min. T cells in the starting and depleted populations were detected by staining with biotinylated monoclonal anti-CD3 (Pharmingen) and then with fluorescein isothiocyanate-conjugated streptavidin. Fewer than 2% of the T-depleted cells stained with the T-cell marker compared with 33% of control splenocytes.

Lymphocyte proliferation assays. Spleens were obtained from naive mice, and single-cell suspensions were prepared by gentle disruption. Unfractionated lymphocytes, T-depleted splenocytes, or B-depleted splenocytes were incubated at 2×10^5 per well in 0.2 ml of lymphocyte medium (LM) composed of RPMI containing 1% volume 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 in 96-well microtiter plates. Dilutions of all stimuli were made in LM. Cultures were incubated for 72 h at 37°C under a humidified atmosphere of 5% CO_2. A total of 1 μCi of [^{3}H]thymidine (Amersham) was added to each well for the last 20 h of culturing, and incorporation into DNA was determined from samples collected with a PHD cell harvester (Cambridge Technologies).

Immunoglobulin production by splenocyte cultures. Splenocytes were cultured at a concentration of 10^6/ml for 6 days

in LM, and the supernatants were assayed for immunoglobulin production by antibody capture enzyme-linked immunosorbent assay (ELISA) (12). Microtiter plates were coated with goat antibody to mouse IgG, IgM, and IgA (Zymed), dilutions of supernatants were added to wells, unbound sample was washed off, and the bound murine immunoglobulin was detected by horseradish peroxidase-conjugated antibodies to murine IgG and IgM (Boehringer Mannheim). Immunoglobulin levels were estimated from a standard curve determined with known concentrations of IgG or IgM. The plates were developed by incubation with 0.4 mg of *o*-phenylenediamine per ml and 0.01% H_2O_2 and read at an optical density of 492 nm with a V_{max} 96-well microtest plate spectrophotometer (Molecular Devices).

Cytokine production by splenocyte cultures. Splenocytes were cultured at a concentration of 10^7/ml for 24 h in LM with the indicated stimuli. Supernatants were assayed for IL-6 production by antibody capture ELISA.

Endothelial cells. Human umbilical vein endothelial cells (HUVECs) were isolated from human umbilical cords by collagenase digestion following published procedures (23) and were cultured in endothelial growth medium (Clonetics) on gelatin-coated tissue culture flasks. HUVECs from confluent primary cultures were plated at 2×10^5/ml in 24-well plates in endothelial growth medium. After overnight incubation, purified OspA or OspB was added for a 24-h incubation. Supernatants were tested for IL-6 production.

Bone marrow macrophages. Bone marrow cells were obtained from mouse femurs (31) and suspended in RPMI containing 30% L929 conditioned media, 20% horse serum (Hyclone), 0.05 mM 2-mercaptoethanol, 2 mM L-glutamine, and 0.01 mg of gentamicin sulfate per ml. Bone marrow cells were cultured for 5 to 6 days at 37°C under a humidified atmosphere of 5% CO_2 in petri dishes (Falcon). The plates were rinsed with media, and the adherent macrophage population was recovered by incubation with 10 ml of cold PBS for 20 min. Macrophages were replated in 24-well dishes at a density of 3×10^5 per well in the LM. Macrophages were allowed to adhere by overnight incubation at 37°C, and the indicated stimuli were added. Supernatants were harvested at 24 h, and the levels of IL-6 and TNF-α in the supernatants were determined by ELISA. Recombinant murine gamma interferon (INF-γ) was generously provided by Genentech.

ELISA assay for murine IL-6 and TNFα. In the IL-6 assay the capture antibody was MP5-20F3 (Pharmingen) and the developing antibody was biotin-conjugated MP5-32C11 (Pharmingen [42]). Recombinant IL-6 (R & D Systems) was used for the standard curve. The capture and developing antibodies for the TNF-α ELISA were MP6-XT3 and MP6-XT22 (Pharmingen), respectively, and recombinant TNF-α (Pharmingen) was used as a standard. Samples and standards were diluted in PBS containing 10% fetal bovine serum and incubated overnight at 4°C in the antibody-coated wells. Plates were developed as described above.

Human IL-6. IL-6 was measured in supernatants from human endothelial cells by antibody capture ELISA. The plates were coated with anti-human IL-6 monoclonal antibody (Genzyme) for capture, and IL-6 in samples was detected with goat anti-human IL-6 (R & D Systems) and biotinylated rabbit anti-goat IgG. Plates were incubated with horseradish peroxidase-conjugated avidin and developed as described above. The recombinant human IL-6 standard was a gift of William B. Ershler, University of Wisconsin.

RESULTS

Initial experiments to define the biochemical properties of the *B. burgdorferi* B-cell mitogen and cytokine stimulatory molecules involved proteolytic digestion and chloroform extraction. Both activities in the crude sonicate were completely resistant to digestion with proteinase K, although all protein bands disappeared by SDS-polyacrylamide gel electrophoresis (PAGE) analysis. Following digestion, the proportion of activity recovered in the chloroform phase of a Bligh and Dyer chloroform-methanol-water (5:10:4) extraction (11) increased from 30 to 60% (data not shown). The sonicate was subjected to 100,000 × *g* centrifugation, after which approximately 60% of both of these activities was found in the pellet, suggesting association with membrane fractions of *B. burgdorferi*. Together these findings suggested the activities were associated with a fatty acid modification of a membrane protein such as a lipoprotein. A report by Radolf et al. (35), demonstrating that outer surface lipoproteins of both *B. burgdorferi* and *Treponema pallidum* could stimulate TNF-α promoter activity in macrophages transfected with a reporter construct further directed our attention to the outer surface lipoproteins of *B. burgdorferi*, specifically the well-characterized molecules OspA and OspB.

OspA and OspB were purified from the *n*-butanol extract of the membrane-enriched fraction of sonicated *B. burgdorferi* N40. The nonionic detergent *n*-octyl-β-glucoside was added to the aqueous fraction of the *n*-butanol extract before application to nonspecific precolumns and to anti-OspA and OspB monoclonal antibody affinity columns. As shown in the silver-stained gel in Fig. 1, OspA and OspB were purified from other contaminating proteins; however, preparations of each were still slightly contaminated with the other lipoprotein. This suggests there is a specific association between a small proportion of these lipoproteins. A *B. burgdorferi* variant of the HB19 strain that was deficient in OspB was provided by Alan Barbour. This allowed purification of OspA that was totally free of OspB (Fig. 1). There was no evidence in silver-stained gels for the presence of low-molecular-weight lipoproteins or other contaminants such as LPS in any of these preparations (Fig. 1).

A major concern was that during the purification of OspA or OspB there would be opportunities for contamination with bacterial endotoxin. Precautions were taken at each step to eliminate sources of contamination by baking glassware used for dialysis and buffer preparation, sequestering all chemicals used in the purification, using water that had been passed through an endotoxin filter, and soaking heat-sensitive equipment in an endotoxin-neutralizing solution, E-Toxa-Clean. A *Limulus* amebocyte lysate assay sensitive to 0.03 EU was used to test endotoxin levels in the purified OspA and OspB and demonstrated these had less than 0.03 EU/500 ng. OspA and OspB did not inhibit the endotoxin standard. The tissue culture medium was slightly contaminated, 0.4 ng/ml; however this level appeared to have no effect on lymphocytes and macrophages used in these assays and would be readily inhibited by the polymyxin B added to cultures (34). Even though medium was only slightly contaminated with endotoxin, polymyxin B was added to the cultures to control for inadvertent contamination of murine cells during preparation. Although not shown in all figures, B-cell mitogen assays always included controls of 1 μg of commercial LPS per ml in the presence and absence of polymyxin B. This control established the health of cells used in these assays and the activity of the polymyxin B,

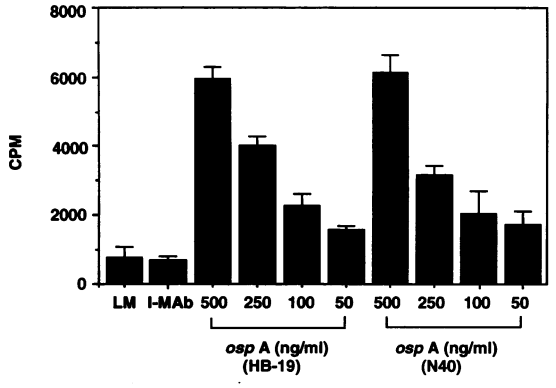


FIG. 2. Mitogenic activity of OspA purified from different strains of *B. burgdorferi*. OspA purified from the HB-19 or N40 strains of *B. burgdorferi* was tested for the ability to stimulate proliferation of splenocytes from C3H/HeJ mice. The concentration of OspA is indicated with all dilutions made in LM. LM stands for LM alone, and I-MAb stands for the eluate of an irrelevant monoclonal antibody column; the volume shown is equivalent to that used for the highest concentration of OspA. Proliferation was measured in 3-day cultures by the incorporation of [^{3}H]thymidine during the last 20 h. Values represent the means and standard deviations of triplicate samples.

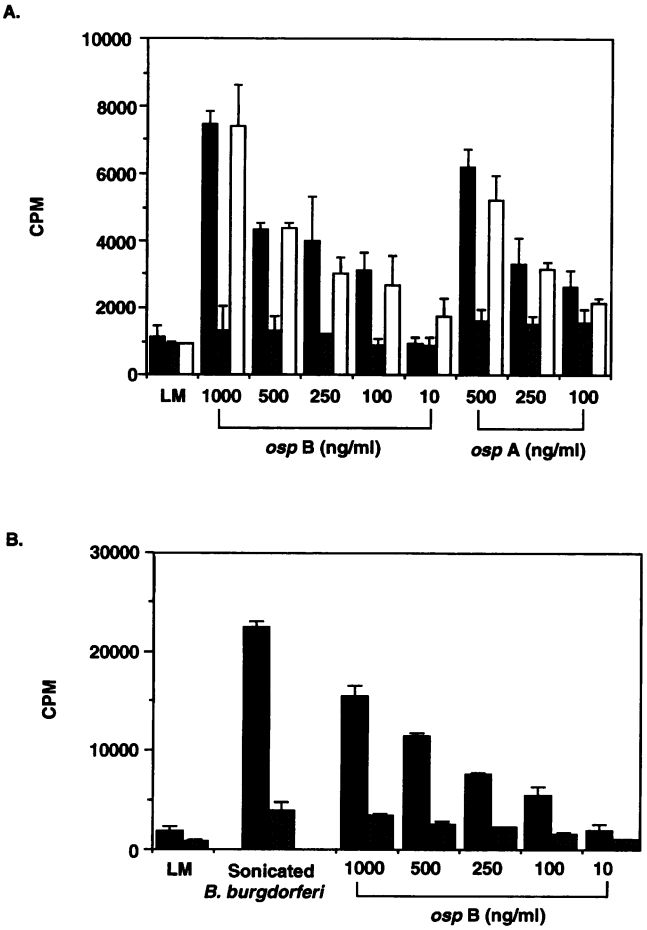


FIG. 3. Proliferative response of splenocytes to OspA and OspB. Unfractionated (filled bars), B-depleted (hatched), or B-enriched (open bars) splenocytes were incubated at a concentration of 2×10^5 per well with LM alone or with the indicated concentrations of purified OspA or OspB, both purified from the N40 strain. Sonicated N40 *B. burgdorferi* was at 5 μg/ml. Splenocytes were from BALB/c mice (A) or C3H/HeJ mice (B). Proliferation was measured as for Fig. 2.

which normally inhibited 1 μg of LPS per ml by greater than 90%. Cells from BALB/c mice responded intensely, while cells from the endotoxin-resistant C3H/HeJ mice responded weakly to this high concentration of LPS. In several experiments, lipoprotein-treated samples were incubated in the presence and absence of polymyxin B. Results were always similar, indicating that synergism with very low levels of LPS was not required for activity of the lipoproteins.

OspA purified from both strains of *B. burgdorferi* was tested for the ability to stimulate proliferation of splenocytes from naive C3H/HeJ mice. Potent stimulatory activities were associated with both preparations, with similar dose-response characteristics (Fig. 2). An eluate from the irrelevant monoclonal antibody column was also tested for stimulatory activity and was found to be inactive at several dilutions. The highest concentration tested, with a volume equivalent to that of 500 ng of OspA per ml from HB19, is shown in Fig. 2. This indicates that the affinity chromatography step does not cause concentration of some unrelated bioactive material and further supports the conclusion that the mitogen activity is specifically associated with the highly purified lipoproteins. Furthermore, the activity of OspA purified from two distinct strains is similar. This suggests that the association between a small proportion of OspA and OspB molecules is not required for activity, as OspA purified from the OspB-negative strain is as active as OspA from N40 (Fig. 2). C3H/HeJ mice are resistant to bacterial endotoxin, further indicating that this activity is independent of LPS contamination. Similar results were obtained with splenocytes from BALB/c mice (Fig. 3). The high degree of purity of these molecules as demonstrated in the silver-stained gel in Fig. 1 provides strong evidence that these activities are properties of OspA and OspB and not of a trace contaminant. The experiments discussed in the rest of the paper were performed several times, with the two OspA preparations giving similar results. Typical experiments are shown in the remaining figures, displaying the activity of one or the other OspA preparation. The possibility that residual detergent or other inhibitory activities were found in the samples was addressed by mixing the purified lipoproteins with stimulatory agents. OspB at up to 500 ng/ml had a minimal inhibitory effect on proliferative responses to mitogens such as concanavalin A and anti-CD3. High concentrations (500 ng/ml) of OspA did have slight inhibitory activity, perhaps reflecting the larger volume of material required to achieve the high concentration. It is possible that this causes some underestimation of the activity of OspA.

Splenocytes from BALB/c mice also responded to the mitogenic activity of OspA (Fig. 3), and purified OspB was a potent mitogen for splenocytes from BALB/c and C3H/HeJ mice. The dose responses to OspA and OspB were in similar ranges (Fig. 3A). This suggests that the biological properties of OspB were not solely due to the presence of small amounts of OspA. To determine which population of splenocytes was responding to the mitogenic stimulus, cell populations were depleted. Greater than 90% depletion of B cells was achieved by panning on anti-immunoglobulin-coated plates. T cells were depleted by greater than 90% by in vivo depletion with anti-CD4 and CD8 and in vitro lysis with anti-Thy-1.2 and complement. B-cell-depleted popula-

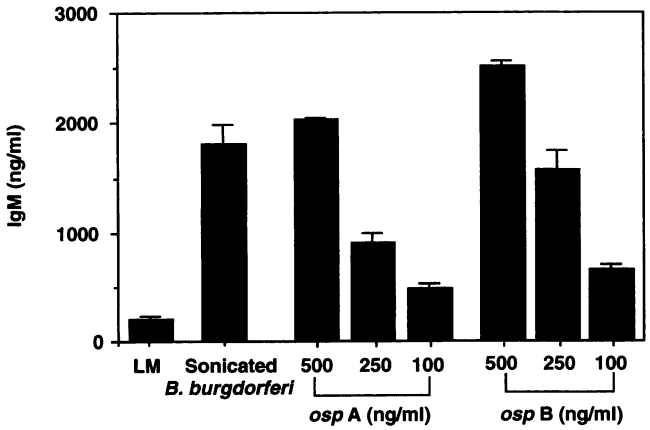


FIG. 4. IgM production by splenocytes incubated with sonicated *B. burgdorferi*, purified OspA, or purified OspB. Splenocytes from C3H/HeJ mice were cultured for 6 days at a concentration of 2×10^5 per well in the presence of LM alone, 0.5 μg of sonicated *B. burgdorferi* per ml, or the indicated concentration of OspA or OspB, both purified from the N40 strain. IgM in the supernatant was detected by antibody capture ELISA. Values represent the means and standard deviations of triplicate samples.

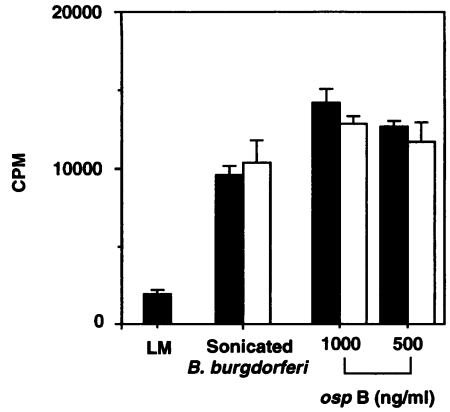


FIG. 5. Effects of proteinase K digestion on the mitogenic activity of sonicated *B. burgdorferi* and OspB. Sonicated *B. burgdorferi* (1 μg/ml) or purified OspB was subjected to digestion with proteinase K at 37°C (open bars) or incubated at 37°C in digestion buffer (closed bars), as described in Material and Methods. The treated material was added to splenocyte cultures from C3H/HeJ mice, and proliferation was measured on day 3 as described in the legend to Fig. 2. LM is LM alone.

tions from BALB/c mice (Fig. 3A) and C3H/HeJ mice (Fig. 3B) did not respond to OspA and OspB, whereas the T-cell-depleted population did (Fig. 3A). This is in agreement with our previous finding with the crude sonicate, which was mitogenic for B cells but not T cells (39). The results of Fig. 2 and 3 demonstrate trends typical of many experiments, and it should be noted that day-to-day variation in the absolute magnitude of the response was observed.

Splenocytes from C3H/HeJ mice that were incubated with purified OspA or OspB for 6 days produced IgM, as detected by ELISA in Fig. 4, and as previously reported with the crude sonicate (39). IgG was not detected in culture supernatants. This indicates that OspA and OspB alone can stimulate murine B-cell activation differentiation to immunoglobulin secretion, although other cells present in the splenocyte preparation may contribute to immunoglobulin production. The magnitude of the response to OspA and OspB is similar to that obtained with 500 ng of the crude sonicate per ml. The proliferation and immunoglobulin production responses of B lymphocytes from naive mice support the conclusion that OspA and OspB are involved in the polyclonal B-cell activation previously demonstrated with sonicated *B. burgdorferi*.

As mentioned, the initial biochemical characterization indicated that the mitogen activity was not sensitive to proteinase K digestion. Shown in Fig. 5 is the effect of proteinase K digestion on the mitogenic activity of purified OspB. OspB was incubated for 6 h at 37°C in the presence or absence of proteinase K. The mitogenic activity of the digest and control were tested with splenocytes from C3H/HeJ mice and found to be identical. Silver staining of samples analyzed on a SDS–12% polyacrylamide gel indicated that the only protein remaining after the digestion was proteinase K. Phenylmethylsulfonyl fluoride-treated proteinase K had no stimulatory activity in this assay. Thus, the majority of the polypeptide portion of the lipoprotein was not required for mitogenic activity.

Sonicated *B. burgdorferi* and intact organisms have previously been found to stimulate cytokine production by human and animal cells, suggesting that cytokine stimulatory activities could also reside in these lipoproteins. We looked at the effect on splenocytes from naive C3H/HeJ mice and found profound stimulation of IL-6 production. Both OspA and OspB stimulated IL-6 production, with similar dose responses and magnitudes, whereas the eluate from the irrelevant monoclonal antibody column had no effect (Fig. 6). The response was equal to or greater than to an equivalent concentration of sonicated *B. burgdorferi*. IL-6 production was assessed because very high levels of circulating IL-6 were found in C3H/HeJ mice infected with *B. burgdorferi* (48). These findings are indicative of cytokine stimulatory potential in general and are not meant to suggest that only IL-6 was produced.

Several cell types in the spleen are capable of producing IL-6 under different conditions, including T cells and B cells; however, the most likely cell type responsible for this response is the monocyte (46). To further dissect this response, it was desirable to study a relatively pure macrophage population. Bone marrow macrophages were used to assess the ability of cells of the monocyte lineage to respond to OspA and OspB. As shown in Fig. 7 and 8, bone marrow-derived macrophages from C3H/HeJ and BALB/c mice produce inflammatory cytokines when incubated with OspA and OspB. This response was not inhibited by polymyxin B, demonstrated in Fig. 7, which is important in these experiments because of the extreme sensitivity of macrophages to very low concentrations of endotoxin. The polymyxin B was active, as demonstrated by its ability to completely inhibit the response to LPS by BALB/c mice (Fig. 7B). Macrophages from C3H/HeJ mice respond poorly to LPS, seen in the lack of response to 100 ng of LPS per ml (Fig. 7A). Macrophages from the endotoxin-resistant C3H/HeJ mice responded to doses of OspA and OspB similar to those stimulatory for macrophages from the endotoxin-responsive BALB/c mice. Extremely high levels (up to 800 ng/ml) of IL-6 were produced by macrophages incubated with OspA or OspB. The results shown in Fig. 7 are typical of several experiments, and although day-to-day experimental variability in the amount of IL-6 produced was observed (Fig. 7A and C), there was no consistent difference in the

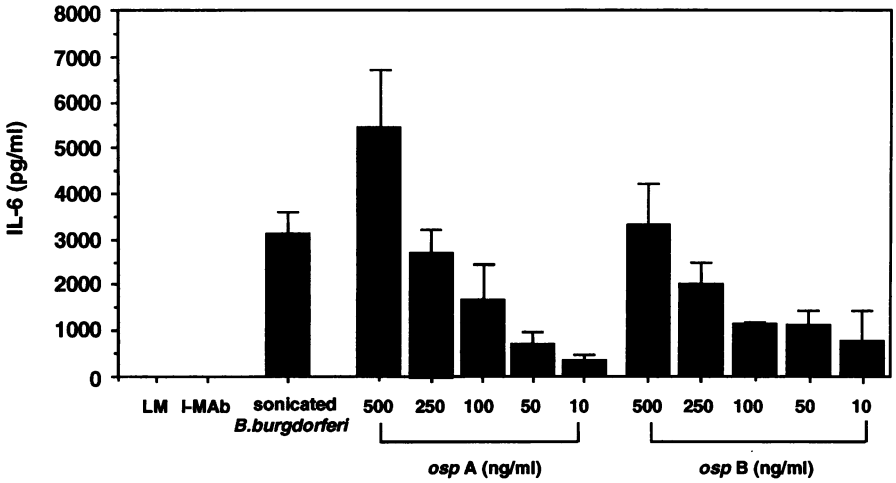


FIG. 6. IL-6 production by splenocytes cultured with sonicated *B. burgdorferi* or purified OspA or OspB. Splenocytes (2×10^6) from C3H/HeJ mice were incubated with 0.5 μg of sonicated *B. burgdorferi* per ml or the indicated concentration of OspA or OspB, purified from N40. LM represents samples incubated with LM alone, and I-MAb represents samples incubated with the eluate of an irrelevant monoclonal antibody column at a volume equal to that of the highest concentration of OspA. IL-6 levels in the supernatant were determined at 24 h by antibody capture ELISA. Values represent the means and standard deviations of triplicate samples.

amount of IL-6 produced by macrophages from the two strains of mice. The macrophage supernatants from Fig. 7 were also assayed for the presence of TNF-α (Fig. 8). OspA and OspB did stimulate the production of TNF-α by macrophages, although at levels lower than for IL-6 (Fig. 8). Macrophages from C3H/HeJ mice consistently produced less TNF-α than did macrophages from BALB/c mice, which could possibly be related to the low TNF-α response responsible for endotoxin resistance in C3H/HeJ mice. The addition of 1 U of IFN-γ per ml to the macrophage cultures caused no cytokine production on its own, but caused approximately a twofold enhancement of IL-6 and TNF-α production by macrophages (Fig. 7 and 8). Eluate from an irrelevant monoclonal column had no cytokine stimulatory activity.

The results with bone marrow-derived macrophages indicate that nonlymphoid cells respond to the purified lipoproteins of *B. burgdorferi*. The extremely high IL-6 response coupled with its presence in infected arthritic mice suggests that such potent cytokine stimulatory activities are involved in inflammatory events of infected tissue. As invasion of these tissues appears to require interaction with vascular endothelium, we assessed whether endothelial cells could also respond to OspA and OspB. Low, but significant, levels of IL-6 were produced by HUVECs from two different donors when incubated with sonicated *B. burgdorferi* or purified OspA and OspB (Fig. 9). Thus, a mechanism of localized inflammatory stimulation at sites of invaded tissues could result from the cytokine stimulatory lipoproteins.

One surprising observation was that the maximal response to purified OspA and OspB was achieved at 500 to 1,000 ng/ml, best demonstrated in Fig. 7C, whereas the response to the crude sonicate was maximal at 5 μg/ml (39). Although we might have expected greater activity of the purified moieties, we do not know if this means that aggregates found in the crude sonicate are more active than the detergent-purified material or if there are other molecules contributing to the activity of the crude sonicate.

DISCUSSION

To determine whether the major outer surface lipoproteins were responsible for the mitogen and cytokine stimulatory activities previously identified in sonicated *B. burgdorferi*, it was necessary to develop an efficient protocol for purification of these lipoproteins from the spirochete. OspA was purified from two different strains of *B. burgdorferi*, the N40 strain and an OspB-deficient variant of HB19. Both preparations were homogeneous by silver stain analysis (Fig. 1), and none of the preparations were contaminated with bacterial endotoxin. The purification of OspA from the OspB-negative strain completely eliminated the possibility of contamination with small amounts of OspB. The two OspA preparations had equivalent biological activities; thus, there was no requirement for coassociation with OspB for activity. OspB was purified from the N40 strain and was still slightly contaminated with OspA, suggesting a tight association between these lipoproteins, as reported by others (35). The biological activities of OspB were in the same dose range as those of OspA, indicating that both lipoproteins had mitogenic and cytokine stimulatory activities and that the presence of OspA was not responsible for the activity of OspB. The coassociation of OspB with OspA suggests that the lipoproteins form aggregates, even in the presence of detergents added during affinity chromatography. The *n*-octyl-β-glucoside added to the samples during the chromatography to remove free phospholipids did not dissociate complexes of OspA and OspB, nor did a variety of other detergents added singly or in combinations. The ability of OspA and OspB to form tight complexes with each other suggests that the lipoproteins could form complexes by themselves also. Whether the OspA and OspB purified by these procedures was in monomeric or multimeric form was not addressed in these studies.

Purified OspA and OspB possessed both the B-cell mitogen and cytokine stimulatory activities. OspA and OspB stimulated proliferation of B lymphocytes, but not T lymphocytes (Fig. 3) and stimulated IgM production by splenocytes from naive mice (Fig. 4). OspA and OspB also stimu-

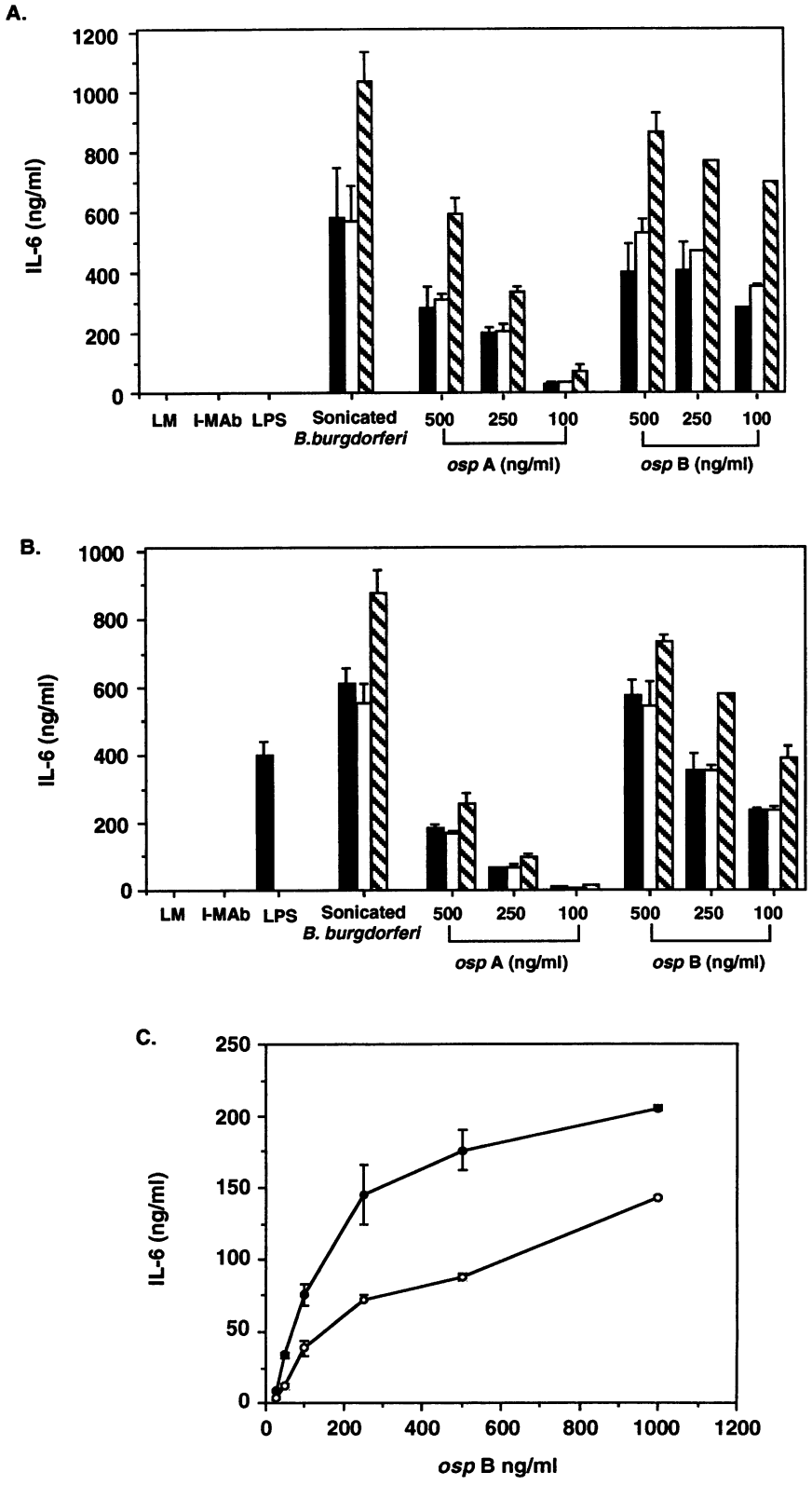
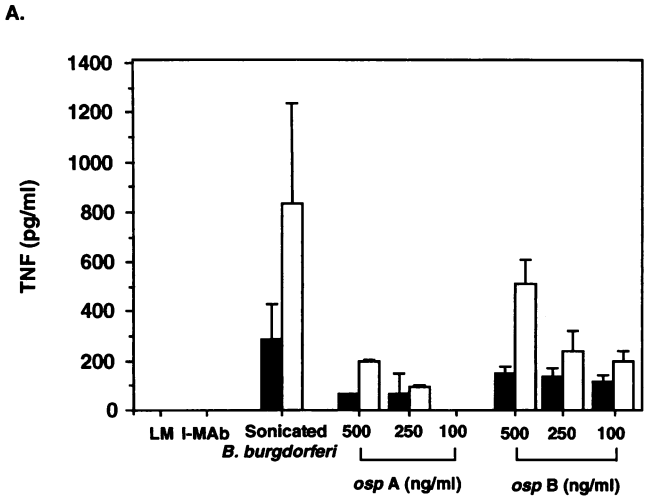


FIG. 7. IL-6 production by bone marrow-derived macrophages incubated with OspA or OspB. Bone marrow-derived macrophages from C3H/HeJ mice (A) or BALB/c mice (B) were plated at 3×10^5 per well in 24-well cluster dishes, and samples were incubated with the indicated concentrations of OspA (from HB19), OspB (N40), or 5 μg of sonicated *B. burgdorferi* per ml. LM is LM alone, I-MAb is the eluate from an irrelevant monoclonal antibody column, and LPS is commercial bacterial endotoxin at 100 ng/ml. Closed bars indicate samples incubated in LM without polymyxin B. Open bars contain 10 μg of polymyxin B per ml, and hatched bars contain polymyxin B and 1 U of IFN-γ per ml. (C) A more complete dose response of macrophages from C3H/HeJ mice to OspB. Samples were incubated in the presence (filled circles) or absence (open circles) of 1 U of IFN-γ per ml. (A to C) Supernatants were harvested at 24 h and assayed for the presence of IL-6. Values represent the means and standard deviations of duplicate samples.



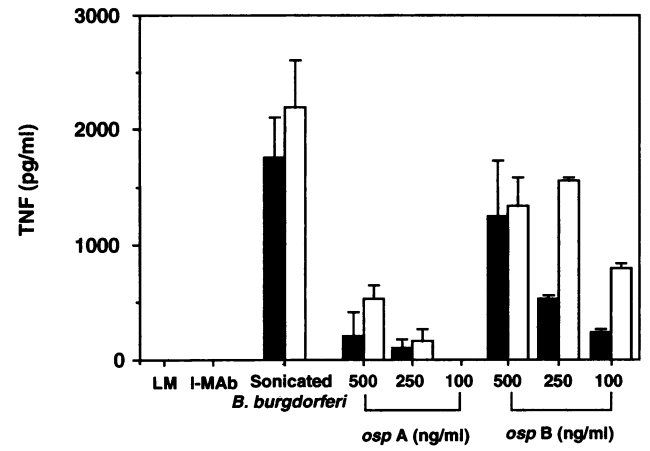


FIG. 8. TNF-α production by bone marrow-derived macrophages incubated with OspA or OspB. Supernatants from the bone marrow-derived macrophage cultures described in the legend to Fig. 7 were assayed for the presence of TNF-α by antibody capture ELISA. Macrophages were from C3H/HeJ mice (A) or BALB/c mice (B). Cultures were incubated without (filled bars) or with (open bars) 1 U of INF-γ per ml. Values represent the means and standard deviations of duplicates.

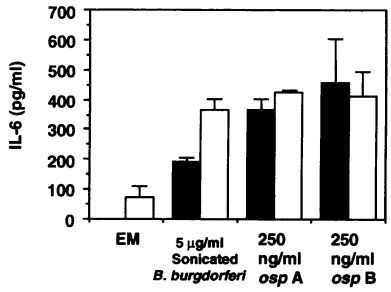


FIG. 9. Sonicated *B. burgdorferi*, OspA, and OspB stimulate human endothelial cells to produce IL-6. HUVECs were plated in 24-well cluster dishes and incubated with endothelial cell medium alone (EM), 5 μg of sonicated *B. burgdorferi* per ml, or 250 ng of OspA (HB19) or OspB (N40) per ml. Supernatants were harvested at 24 h, and IL-6 in the supernatant was determined by antibody capture ELISA for human IL-6, as described in Material and Methods. Values represent the means and standard deviations of duplicate samples. Filled and open bars represent values with HUVECs from two different donors.

lated IL-6 production by splenocytes (Fig. 6). Bone marrow-derived macrophages produced high levels of IL-6 and significant levels of TNF-α in response to OspA and OspB (Fig. 7 and 8), indicating cells of this lineage can also respond to these lipoproteins. These findings are consistent with those of Radolf et al. (35), in which lipoproteins purified from *B. burgdorferi* and *T. pallidum* were found to stimulate TNF-α promoter activity in transfected cell lines. Dose-response ranges were similar for OspA and OspB in most assays, and for cells from C3H/HeJ and BALB/c mice. Comparison of the dose response of OspA and OspB with the activity of the crude sonicate is surprising, as the crude sonicate is very active, and we have not achieved the degree of enrichment in activity we would expect on the basis of the tremendous purification. This could be due to a more active presentation of OspA and OspB in the crude sonicate, perhaps by aggregation, or it could indicate that other molecules in the sonicate contribute to its bioactivity.

There was no apparent difference in the response to OspA or OspB in cells from BALB/c and C3H/HeJ mice that could explain the dramatic difference in arthritis found in the two strains. The addition of INF-γ to macrophage cultures enhanced the production of IL-6 and TNF-α by approximately twofold (Fig. 7 and 8). This is an interesting observation, as it suggests that the level of various cytokines in the microenvironment of inflamed tissue could influence the magnitude of the response of resident cells to borrelia-associated OspA and OspB in vivo, whereas the potential to respond measured in vitro is quite similar. This may have implications in the murine model, where there is a well-documented predisposition for the production of IFN-γ by C3H/HeJ mice and lack of INF-γ production in BALB/c mice (30). During infection with *B. burgdorferi*, severely arthritic C3H/HeJ mice have very high elevation in serum IL-6, while mildly arthritic BALB/c mice have barely detectable serum IL-6.

Another important cell type with which *B. burgdorferi* interacts during infection and tissue invasion is the endothelial cell. *B. burgdorferi* can transcytose endothelial monolayers in vitro (13, 44), and it is proposed that this is the mechanism by which the spirochete gains access to various tissues. *B. burgdorferi* can also gain an intracellular location within endothelial cells cultured in vitro (14, 28). The intracellular location could provide a protective niche for the spirochete; however, we have not been able to recover viable spirochetes from within endothelial cells, nor have we found evidence for intracellular multiplication (unpublished observations). Endothelial cells participate in inflammatory responses by the expression of adhesion molecules and class II molecules, and by the production of cytokines (29). Purified OspA and OspB did stimulate IL-6 production by endothelial cells from two different donors (Fig. 9). Similar results have recently been reported with *T. pallidum* (36).

Outer surface lipoproteins of *E. coli* and other gram-negative bacteria have been found to possess mitogenic and cytokine stimulatory activities (7, 21). Interestingly, in our initial report, we compared the activities of sonicated *E. coli* with sonicated *B. burgdorferi* and found no activity in the *E.*

coli sonicate that was resistant to polymyxin B (39). This was an indication that *B. burgdorferi* possessed an activity with unique potency, as *E. coli* and other gram-negative bacteria possess biologically active lipoproteins. The *E. coli* Braun lipoprotein is the prototype of bacterial lipoproteins and has been demonstrated to have B-cell mitogenic properties and the ability to stimulate cytokine production by macrophages (7, 21, 22, 32). The Braun lipoprotein possess three fatty acid residues attached to the amino-terminal cysteine. Two of the fatty acids are ester linked, and the third is amide linked (32). The genes encoding OspA and OspB include sequences coding for the prokaryotic consensus sequence for amino-terminal lipid modification (6). Brandt et al. have demonstrated that OspA and OspB can be labeled with [^{3}H]-palmitate, suggesting fatty acid modifications and a structure similar to *E. coli* lipoproteins (8). The retention of activity by OspB following proteinase K digestions (Fig. 5) further suggests that the fatty acid-modified region of the protein is responsible for activity. Studies with the Braun lipoprotein (M_r, approximately 6500) reported maximal bioactivity at 5 μg/ml (32). Synthetic lipopeptides based on this structure, with an M_r of approximately 1,200, have also been studied, and the maximal biological responses of the most active are achieved at 10 μg/ml (22). The activity of OspB and OspA, containing molecular species of 34,000, and 31,000 Da, respectively, plateaued in the range of 500 ng/ml, most clearly demonstrated in Fig. 7C, indicating that these molecules are much more potent than those previously studied. On the basis of a molar comparison the potency for both OspA and OspB is approximately 50-fold greater than for the Braun lipoprotein and 500-fold greater than for the synthetic lipopeptides that have been studied. A report by de Souza et al. found that 10 μg of recombinant OspA per ml in the form of a glutathione-transferase fusion protein had mitogenic activity that was only partially inhibited with polymyxin B (16). Dose-response information is not available on this recombinant, making it difficult to compare with the activity of the native protein isolated from *B. burgdorferi*. The biochemical feature responsible for the unique potency of lipoproteins purified from *B. burgdorferi* will be the subject of future studies. This understanding may be very important, as in animal vaccine trials recombinant OspA lipoprotein has been found to be a more potent immunogen than a nonlipidated recombinant (18).

The finding that purified OspA and OspB stimulate polyclonal B-cell activation and cytokine production by macrophages and endothelial cells may be important for pathological developments in vivo. Both of these properties do function in *B. burgdorferi*-infected mice (48). The invasion of joints and cardiac tissue by the spirochete would allow introduction of these potent molecules into the tissue (4). The interaction of OspA and OspB with resident cells such as the macrophage-like cells of the synovium could cause cytokine production, proliferation, and recruitment of inflammatory cells, leading to the development of arthritis. This supports a model in which three factors contribute to arthritis development: (i) invasion of tissues and persistence in tissues by the spirochete, (ii) interaction of the stimulatory molecules OspA and OspB with responsive cells, and (iii) modulation of the effect of OspA and OspB by cytokines produced in the microenvironment of the invaded tissues. The final factor may vary from individual to individual, or mouse strain to mouse strain, and may provide insight as to the range of symptoms observed with *B. burgdorferi* infection.

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REFERENCES

1. **Barbour, A. G.** 1984. Isolation and cultivation of Lyme disease spirochetes. Yale J. Biol. Med. **57**:521.
2. **Barbour, A. G., S. L. Tessier, and W. J. Todd.** 1983. Lyme disease spirochetes and ixodid tick spirochetes share a common surface antigenic determinant defined by a monoclonal antibody. Infect. Immun. **41**:795–804.
3. **Barthold, S. W., D. S. Beck, G. M. Hansen, G. A. Terwilliger, and K. D. Moody.** 1990. Lyme borreliosis in selected strains and ages of laboratory mice. J. Infect. Dis. **162**:133–138.
4. **Barthold, S. W., D. H. Persing, A. L. Armstrong, and R. A. Peeples.** 1991. Kinetics of *Borrelia burgdorferi* dissemination and evolution of disease after intradermal inoculation of mice. Am. J. Pathol. **139**:263–273.
5. **Beck, G., J. Benach, and G. S. Habicht.** 1989. Isolation of interleukin 1 from joint fluids of patients with Lyme disease. J. Rheumatol. **16**:800–806.
6. **Bergstrom, S., V. G. Bundoc, and A. G. Barbour.** 1989. Molecular analysis of linear plasmid-encoded major surface proteins, OspA and OspB, of the Lyme disease spirochaete *Borrelia burgdorferi*. Mol. Microbiol. **3**:479–486.
7. **Bessler, W. G., M. Cox, A. Lex, B. Suhr, K. Wiesmuller, and G. Jung.** 1985. Synthetic lipopeptide analogs of bacterial lipoprotein are potent polyclonal activators for murine B lymphocytes. J. Immunol. **135**:1900–1905.
8. **Brandt, M. E., B. S. Riley, J. D. Radolf, and M. V. Norgard.** 1990. Immunogenic integral membrane proteins of *Borrelia burgdorferi* are lipoproteins. Infect. Immun. **58**:983–991.
9. **Bundoc, V. G., and A. G. Barbour.** 1989. Clonal polymorphisms of outer membrane protein OspB of *Borrelia burgdorferi*. Infect. Immun. **57**:2733–2741.
10. **Burgdorfer, W., A. G. Barbour, S. F. Hayes, J. L. Benach, E. Grunwaldt, and J. P. Davis.** 1982. Lyme disease—a tick-borne spirochetosis? Science **216**:1317–1319.
11. **Chrisite, W. W. (ed.).** 1982. Lipid analysis, 2nd ed. Pergamon Press, New York.
12. **Coligan, J. E., A. M. Kruisbeek, D. H. Margulies, E. M. Shevach, and W. Strober (ed.).** 1991. Current protocols in immunology. John Wiley & Sons, Inc., New York.
13. **Comstock, L. E., and D. D. Thomas.** 1989. Penetration of endothelial cell monolayers by *Borrelia burgdorferi*. Infect. Immun. **57**:1626–1628.
14. **Comstock, L. E., and D. D. Thomas.** 1991. Characterization of *Borrelia burgdorferi* invasion of cultured endothelial cells. Microb. Pathog. **10**:137–148.
15. **Defosse, D. L., and R. C. Johnson.** 1992. In vitro and in vivo induction of tumor necrosis factor alpha by *Borrelia burgdorferi*. Infect. Immun. **60**:1109–1113.
16. **de Souza, M. S., E. Fikrig, A. L. Smith, R. A. Flavell, and S. W. Barthold.** 1992. Nonspecific proliferative responses of murine lymphocytes to *Borrelia burgdorferi* antigens. J. Infect. Dis. **165**:471–478.
17. **Dialynas, D. P., D. B. Wilde, P. Marrack, A. Peirres, K. A. Wall, W. Havran, G. Otten, M. R. Loken, M. Pierres, J. Kappler, and F. W. Fitch.** 1983. Characterization of the murine antigenic determinant designated L3T4a, recognized by monoclonal antibody GK1.5: expression of L3T4a by functional T cell clones appears to correlate primarily with class II MHC antigen-reactivity. Immunol. Rev. **74**:29–58.

18. **Erdile, L. F., M. Brandt, D. J. Warakomski, G. J. Westrack, A. Sadziene, A. G. Barbour, and J. P. Mays.** 1993. Role of attached lipid in immunogenicity of *Borrelia burgdroferri* Osp A. Infect. Immun. **61**:81–90.
19. **Gondolf, K. B., S. R. Batsford, and A. Vogt.** 1990. Isolation of an outer membrane protein complex from *Borrelia burgdorferi* by *n*-butanol extraction and high-performance ion-exchange chromatography. J. Chromatogr. **521**:325–334.
20. **Habicht, G. S., L. I. Katona, and J. L. Benach.** 1991. Cytokines and the pathogenesis of neuroborreliosis: *Borrelia burgdorferi* induces glioma cells to secrete interleukin-6. J. Infect. Dis. **164**:568–574.
21. **Hauschildt, S., P. Hoffmann, H. U. Beuscher, G. Dufhues, P. Heinrich, K. Wiesmuller, G. Jung, and W. G. Bessler.** 1990. Activation of bone marrow-derived mouse macrophages by bacterial lipopeptide: cytokine production, phagocytosis and Ia expression. Eur. J. Immunol. **20**:63–68.
22. **Hoffmann, P., S. Heinle, U. F. Schade, H. Loppnow, A. J. Ulmer, H. D. Flad, G. Jung, and W. G. Bessler.** 1988. Stimulation of human and murine adherent cells by bacterial lipoprotein and synthetic lipopeptide analogues. Immunobiology **177**:158–170.
23. **Jaffe, E. A., R. L. Nachman, C. G. Becker, and C. R. Minick.** 1973. Culture of human endothelial cells derived from umbilical veins. J. Clin. Invest. **52**:2745–2756.
24. **Kenefick, K. B., J. A. Lederer, R. F. Schell, and C. J. Czuprynski.** 1992. *Borrelia burgdorferi* stimulates release of interleukin-1 activity from bovine peripheral blood monocytes. Infect. Immun. **60**:3630–3634.
25. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
26. **Ledbetter, J. A., and L. A. Herzenberg.** 1979. Xenogeneic monoclonal antibodies to mouse lymphoid differentiation antigens. Immunol. Rev. **47**:63–90.
27. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265–275.
28. **Ma, Y., A. Sturrock, and J. J. Weis.** 1991. Intracellular localization of *Borrelia burgdorferi* within human endothelial cells. Infect. Immun. **59**:671–678.
29. **Mantovani, A., and E. Dejana.** 1989. Cytokines as communication signals between leukocytes and endothelial cells. Immunol. Today **10**:370–375.
30. **Mason, D.** 1991. Genetic variation in the stress response: susceptibility to experimental allergic encephalomyelitis and implications for human inflammatory disease. Immunol. Today **12**:167.
31. **Meerpohl, H. G., M. L. Lohmann-Matthes, and H. Fischer.** 1976. Studies on the activation of mouse bone marrow-derived macrophages by the macrophage cytotoxicity factor (MCF). Eur. J. Immunol. **6**:213–217.
32. **Melchers, F., V. Braun, and C. Galanos.** 1975. The lipoprotein of the outer membrane of *Escherichia coli*: a B-lymphocyte mitogen. J. Exp. Med. **142**:473–482.
33. **Miller, L. C., S. Isa, E. Vannier, K. Georgilis, A. C. Steere, and C. A. Dinarello.** 1992. Live *Borrelia burgdorferi* preferentially activate interleukin-1β gene expression and protein synthesis over the interleukin-1 receptor antagonist. J. Clin. Invest. **90**:906–912.
34. **Morrison, D. C., and D. M. Jacobs.** 1976. Binding of polymyxin B to the lipid A portion of bacteral lipopolysaccharides. Immunochemistry **13**:813–818.
35. **Radolf, J. D., M. V. Norgard, M. E. Brandt, R. D. Isaacs, P. A. Thompson, and R. Beutler.** 1991. Lipoproteins of *Borrelia burgdorferi* and *Treponema pallidum* activate cachectin/tumor necrosis factor synthesis. J. Immunol. **147**:1968–1974.
36. **Riley, B. S., N. Oppenheimer-Marks, E. J. Hansen, J. D. Radolf, and M. V. Norgard.** 1992. Virulent *Treponema pallidum* activates human vascular endothelial cells. J. Infect. Dis. **165**:484–493.
37. **Rosa, P. A., T. Schwan, and D. Hogan.** 1992. Recombination between genes encoding major outer surface proteins A and B of *Borrelia burgdorferi*. Mol. Microbiol. **6**:3031–3040.
37a. **Sădžiene, A.** Personal communication.
38. **Sădžiene, A., P. A. Rosa, P. A. Thompson, D. M. Hogan, and A. G. Barbour.** 1992. Antibody-resistant mutants of *Borrelia burgdorferi*: in vitro selection and characterization. J. Exp. Med. **176**:799–809.
39. **Schoenfeld, R., B. Araneo, Y. Ma, L. Yang, and J. J. Weis.** 1992. Demonstration of a B-lymphocyte mitogen produced by the Lyme disease pathogen, *Borrelia burgdorferi*. Infect. Immun. **60**:455–464.
40. **Sigal, L. H.** 1989. Lyme disease, 1988: immunologic manifestations and possible immunopathogenetic mechanisms. Sem. Arthr. Rheum. **18**:153–167.
41. **Sigal, L. H., A. C. Steere, and J. M. Dwyer.** 1988. In vivo and in vitro evidence of B cell hyperactivity during Lyme Disease. J. Rheumatol. **15**:648–654.
42. **Starnes, H. F., M. K. Pearce, A. Tewari, J. H. Yim, J. Zou, and J. S. Abrams.** 1990. Anti-IL-6 monoclonal antibodies protect against lethal *Escherichia coli* infection and lethal tumor necrosis factor-α challenge in mice. J. Immunol. **145**:4185–4191.
43. **Steere, A. C.** 1989. Lyme Disease. N. Engl. J. Med. **321**:586–596.
44. **Szczepanski, A., M. B. Furie, J. L. Benach, B. P. Lane, and H. B. Fleit.** 1990. Interaction between *Borrelia burgdorferi* and endothelium in vitro. J. Clin. Invest. **85**:1637–1647.
45. **Tedder, T. F., L. T. Clement, and M. D. Cooper.** 1984. Expression of C3d receptors during human B cell differentiation: immunofluorescence analysis with the HB-5 monoclonal antibody. J. Immunol. **133**:678–683.
46. **Van Snick, J.** 1990. Interleukin-6: an overview. Annu. Rev. Immunol. **8**:253–278.
47. **Whitmire, W. M., and C. F. Garon.** 1993. Specific and nonspecific responses of murine B cells to membrane blebs of *Borrelia burgdorferi*. Infect. Immun. **61**:1460–1467.
48. **Yang, L., Y. Ma, R. Schoenfeld, M. Griffiths, E. Eichwald, B. Araneo, and J. J. Weis.** 1992. Evidence for B-lymphocyte mitogen activity in *Borrelia burgdorferi*-infected mice. Infect. Immun. **60**:3033–3041.