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Induction of B-Cell Mitogenesis by Outer Surface Protein C of *Borrelia burgdorferi*

William M. Whitmire, Ph.D. and Claude F. Garon, Ph.D.*

Department of Health and Human Services, Public Health Service, National Institutes of Health, National Institute of Allergy and Infectious Diseases, Laboratory of Vectors and Pathogens, Rocky Mountain Laboratories, Hamilton, Montana

The mitogenic response to OspC in a murine splenic lymphocyte proliferation assay was significantly greater ($p < 0.01$) than the *Borrelia (B.) burgdorferi* flagellin-derived control. Fluorescence-activated cytometry identified the responding cells as B lymphocytes, a finding confirmed by use of specific markers. The 24-kDa borrelial surface protein OspC had been purified by two-dimensional (2D) gel electrophoresis and identified on immunoblots with OspC antisera raised in rabbits. These results indicate that like OspA and OspB, lipoprotein OspC is mitogenic for murine B cells. Sample processing did not appear to contribute to the effect.

Key words: Mitogen, Lipoprotein, Lyme disease, Spirochete

INTRODUCTION

The ability of *Borrelia (B.) burgdorferi*, the etiologic agent of Lyme disease, to cause mitogenesis of murine B cells has been described in several reports (1–4). Recently, we have shown that extracellular membrane blebs, which are shed from spirochetal surfaces, also possess significant B-cell mitogenic activity (5). All of these studies indicate that mitogenesis was not due to lipopolysaccharide (LPS). Moreover, de Souza et al. (3) demonstrated that two recombinant spirochetal lipoproteins, outer surface protein A (OspA) and OspB, were mitogenic. This latter finding was of particular interest since blebs contain significant amounts of OspA, OspB, and a protein of approximately 24 kDa that appears to be the OspC lipoprotein (5–9). It seemed possible that OspC might also contribute to the mitogenic effect of both blebs and whole spirochetes.

MATERIALS AND METHODS

Bacteria and antigen preparations. Low-passage (P6 to P8) strain Sh-2-82 of *B. burgdorferi*, which originated from adult *Ixodes scapularis* (*I. dammini*) (10) ticks (Shelter Island, NY), was grown in BSK II culture medium (11) at 34°C. Spirochetes were isolated from the medium after centrifugation at 10,400 g for 30 minutes at 25°C and washed and resuspended in 0.15-*M* phosphate-buffered saline (PBS, pH 7.2) made with pyrogen-reduced water (<1 EU/mL; Milli Q, Millipore Corp., Bedford, MA).

Antigen preparations were produced by subjecting bacterial suspensions described above to three cycles of freeze-thawing at −80°C and sonication for six 15-seconds cycles (at a setting of 4) with a Branson sonicator (Branson Sonic Power Co., Danbury, CT). The resulting sonicate was centrifuged at 12,100 g for 20 minutes at 4°C. Supernatant fractions from the extracts were retained, filter sterilized (0.22-μM porosity), and assayed for total protein (BCA Protein Assay Reagent; Pierce, Rockford, IL). Final preparations were frozen at −80°C until used. Rabbit anti-OspC antiserum was produced by hyperimmunizing a rabbit with SDS-polyacrylamide gel-resolved 24-kDa protein from strain Sh-2-82 of *B. burgdorferi*. The specificity for OspC was confirmed by the reactivity of this antiserum with recombinant OspC (6, 9).

Polyacrylamide gel electrophoresis. Antigen preparations for one-dimensional (1D) analysis were diluted at a 1:1 ratio in double-strength sodium dodecyl sulfate (SDS) electrophoresis treatment buffer and heated for 5 minutes at 95°C, as previously described (5). For two-dimensional (2D) analysis, antigen preparations were solubilized in first dimension solubilizing solution (9.5-*M* urea, 2.0% Triton X-100, 5% beta-mercaptoethanol, 1.6% Bio-Lyte 5/7 ampholyte [Bio-Rad Laboratories, Richmond, CA], and 0.4% Bio-lyte 3/10 ampholyte [Bio-Rad]) for 2 hours at room temperature. The preparation was then centrifuged at 100,000 g for 2 hours at 4°C, and the supernatant fraction (approximately 30-μG total protein per tube) was subjected to electrophoresis for 3.5 hours in first dimension isoelectric focusing (IEF) tube gels, as described by O'Farrell (12) using a Mini Protean II Tube Cell (Bio-Rad). After electrophoresis, tube gels were equilibrated with single-strength SDS electrophoresis treatment buffer solution for 10 minutes at room temperature and applied to the second dimension in 12% SDS-polyacrylamide slab gels. Further electrophoresis at 200 V was performed with a Mini Protean II gel apparatus (Bio-Rad) and the discontinuous buffer system described by Laemmli (13). Antigen preparations (30-μG total protein per lane) that had been solubilized with electrophoresis treatment buffer as well as molecular size standards (Bio-Rad) were added to some slab gels along with tube gels. Following electrophoresis, proteins were visualized by staining with Coomassie blue or immunoblotted.

Immunoblotting for identification of OspC and flagellin. Spirochetal proteins were electrophoretically transferred from 2D slab gels to nitrocellulose sheets (0.1−μM porosity; Schleicher and Schuell, Inc., Keene, NH) in a Mini Trans-Blot Cell (Bio-Rad) for 1.5 hours at 100 V (5). Following transfer, nitrocellulose sheets were incubated in PBS with 0.05% Tween 20 (blocking buffer) overnight to block non-specific binding sites. The sheets were then reacted with rabbit anti-OspC antiserum or monoclonal antibody H9724 (antiflagellin) diluted 1:500 and 1:25 in blocking buffer, respectively, for 2 hours at room temperature. After exposure to horseradish peroxidase-conjugated goat anti-mouse or antirabbit IgG (heavy- and light-chain specific) diluted 1:2500 or 1:1000 in blocking buffer for 1 hour at room temperature, respectively, bound peroxidase activity was detected with peroxidase substrate solution, as previously described (5). The rabbit anti-OspC antiserum and monoclonal antibody H9724 were supplied by Dr. Schwan (Rocky Mountain Laboratories, Hamilton, MT).

Isolation and purification of OspC and flagellin. Two-dimensional gel-resolved protein spots that comigrated with OspC or flagellin bands in 1D gels and that reacted with anti-OspC antiserum or antiflagellin monoclonal antibody, respectively, were excised from 16 two-dimensional gels and pooled. Proteins in the excised spots were eluted with an Electro-Eluter (Bio-Rad), exhaustively dialyzed against 5-mM ammonium bicarbonate with 0.05% SDS and precipitated overnight at −20°C in acetone containing 1-mM hydrochloric acid. Precipitated proteins were then washed twice in cold acetone, vacuum dried, and stored at −20°C. The protein preparations were resuspended in PBS and assayed for total protein prior to use in the lymphocyte proliferation assay. An area on each stained gel, which contained no detectable protein, was also excised and processed in a similar fashion to serve as a background control.

Lymphocyte proliferation assay. Spleens were aseptically obtained from three naive 5-week-old C57BL/10 female mice obtained from a colony at Rocky Mountain Laboratories. Cell suspensions were washed and resuspended at a concentration of 2×10^6 viable cells/mL in RPMI 1640 culture medium supplemented with 20-mM glutamine and 200 U/mL of penicillin. Triplicate cultures were set up in 96-well flat-bottomed microtiter plates (Flow Laboratories, Inc., McLean, VA) by adding 0.1 mL of the cell suspension to wells containing RPMI medium with 20% (v/v) fetal bovine serum (Hyclone Laboratories Inc., Logan, UT) and either 50 μG/mL of LPS mitogen (LPS from *Escherichia coli* 0111:B4; Difco Laboratories, Detroit, MI), 15 μG/mL of purified OspC or flagellin, or 5 μL of background control (equal to volume of OspC preparation used per culture). After 2 days of incubation at 37°C in a humidified 95% air–5% CO_2 atmosphere, the lymphocyte proliferation assay was performed, as previously described (5). The incorporation of [methyl-^{3}H]thymidine (specific activity 6.7 Ci/mmol; NEN Research Products, Du Pont Co., Wilmington, DE) by the cultures was recorded as disintegrations per minute (DPM; counts per minute/counting efficiency). Results of lymphocyte proliferation assays were expressed as increased DPM, defined as test cultures DPM minus background control cultures DPM. Mean increased DPM ± standard error of the mean (SEM) of triplicate OspC- and flagellin-stimulated cultures were calculated. The results were subjected to the Student's *t*-test and single-factor analysis of variance.

Fluorescence-activated cytometric analysis. After 2 days of exposure to OspC or LPS, spleen cell cultures were centrifuged at 300 g for 10 minutes resuspended in 50 μL of fluorescein-conjugated anti-Thy-1.2 or anti-B220 monoclonal antibody in fluorescence-activated cell sorting (FACS) medium (PBS containing 5% [v/v] fetal bovine serum and 10-mM sodium azide), and incubated for 20 minutes on ice, as previously described (5). Cells were then washed twice, resuspending in 200 μL of FACS medium containing propidium iodide (5 μG/mL), and analyzed with a FACStar I fluorescence-activated cell sorter (Becton Dickinson Immunocytometry Systems, San Jose, CA). Fresh unstimulated spleen cells obtained from a naive mouse were treated with a monoclonal antibody against B220 to set limits (boxed area) for detection of blasting cells (data not shown).

RESULTS

Figure 1A shows that 2D gel electrophoresis resolved OspC (double arrows) and flagellin (single arrow) as two large distinct spots that shared alignment with OspC and flagellin bands, respectively, in 1D gels. Outer surface protein A and OspB were not resolved by the 2D gel electrophoresis system used in this study. The reactivity of rabbit anti-OspC antiserum against the 2D gel-resolved OspC on immunoblots is shown in Fig. 1B. The reactive spots immediately to the left of OspC may be due to peptides of OspC that were modified during sample preparation (12), since similar streaking is often noted when monoclonal antibodies to lipoproteins OspA and OspB are used in this fashion. Reactivity of the antiserum to a basic protein in the higher molecular weight region was also evident (Fig. 1B). Mono-

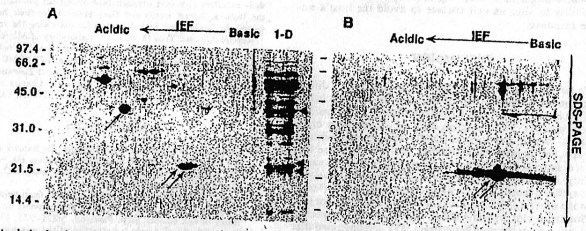

FIG. 1. Analysis of *B. burgdorferi* antigen preparations by 1D and 2D SDS-polyacrylamide gel electrophoresis. (A) The single and double arrows indicate the relative positions of flagellin and OspC, respectively, following 2D gel electrophoresis. Note the alignment of the flagellin and OspC 2D spots with the corresponding (single and double arrowheads, respectively) 1D bands, stained with Coomassie brilliant blue. (B) Immunoblot of an antigen preparation (transferred from a 2D polyacrylamide gel) and reacted with rabbit anti-OspC antiserum. The double arrows indicate the position of the reactive 24-kDa OspC. Positions of molecular size standards are indicated on the left (in kDa).

*Corresponding author

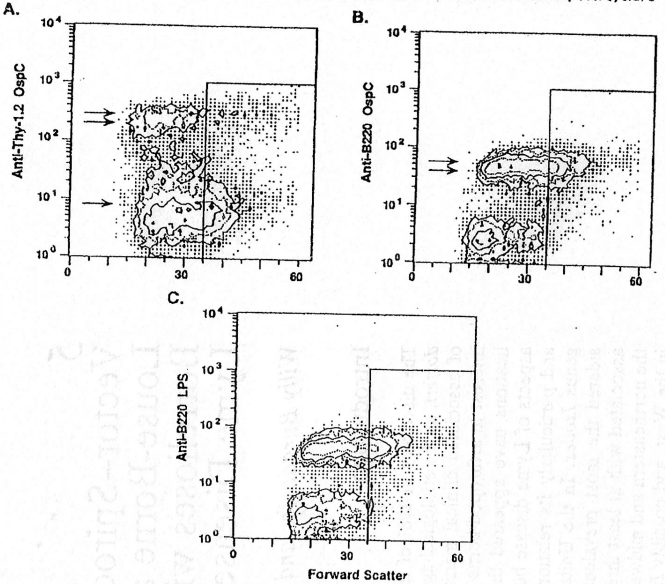

FIG. 2. Fluorescence-activated cytometric analysis of OspC-stimulated murine spleen lymphocytes labeled with monoclonal antibodies directed against Thy-1.2 (A) and B220 (B), representing murine pan-T-cell and murine B-cell markers, respectively. The response of bacterial LPS-stimulated lymphocytes in the presence of anti-B220 is shown in panel C. Single and paired arrows indicate low- and high-fluorescing (labeled) cell populations, respectively, whereas data points in the boxed areas represent blasting (increased cell size) cells. Note that T cells present in OspC-stimulated cultures (A) are predominately small (i.e., lie to left of the boxed area), whereas the fluorescent pattern of OspC- and LPS-stimulated cultures (B and C) are similar following exposure to anti-B220. Lipopolysaccharide is a known mitogen of murine B cells and the B220-positive B cells include many large (blasting) cells after stimulation.

clonal antibody H9724 reacted to the 41 kDa 2D spot (single arrow; Fig. 1A) on immunoblots and identified this spot as flagellin (data not shown). While lymphocytes from naive mice demonstrated significant ($p \leq 0.01$) mitogenic responses to 2D gel-purified OspC in the lymphocyte proliferation assay at 2 days after culture initiation (40,532 ± 4,835 DPM), exposure of lymphocytes to gel-purified flagellin resulted in a low mitogenic response (5195 ± 757 DPM) at equivalent concentrations (15 μG total protein/mL) as OspC. Time course experiments using purified blebs had previously revealed that blastogenic responses after 2 days were similar to or greater than blastogenic responses at 4 or 6 days (data not shown). Analysis of OspC-stimulated spleen cell cultures indicated that OspC-stimulated cells were of the B cell lineage. Blast cells, identified by high forward scatter signal (i.e., cell population within the boxed area) in FACS analysis, were phenotyped as predominately B cells rather than T cells by labeling with the lineage-specific markers B220 and Thy-1.2, respectively (Fig. 2).

DISCUSSION

Two-dimensional gel electrophoresis was used for the isolation of OspC from the Sh-2-82 strain of *B. burgdorferi* because 2D electrophoresis separates proteins by charge as well as by apparent size and can resolve proteins that differ by a single amino acid (12). This greatly reduces the possibility of two or more proteins comigrating as a single spot during electrophoresis (12). The failure of the 2D gel system to resolve OspA and OspB may have been the result of the basicity (>pH 7.0) of these two proteins that prevented them from entering IEF tube gels (pH 6.9 to 5.2) in the first dimension (12, 14). However, immunoblots with rabbit anti-OspC antiserum indicated that the 2D-resolved 24-kDa protein was OspC, since the rabbit antiserum was previously shown to bind to a 24-kDa protein of strain B31 that also reacted with an anti-OspC monoclonal antibody (6, 9). Antiserum reactivity to the higher molecular weight antigen (approximately 40 Kda) may indicate that this antigen shares epitopes with OspC or that this antigen was present within the OspC immunogen used for production of the antiserum. Similar reactivity has also been observed by other investigators on immunoblots of low-passage spirochetes that were exposed to this same rabbit antiserum (6, 9).

Although both time course experiments and dose response curves were described earlier in demonstrating a mitogenic response to purified bleb preparations (5), similar experiments were not possible here given the extremely limited quantities of 2D gel-purified material available. Limited quantities also hampered attempts to obtain an *N*-terminal protein sequence. However, a comparison of equal quantities of 2D gel-purified flagellin and OspC did verify the relatively high mitogenic potential of the 24-Kda protein preparation. Time course and dose response comparisons must await the development of protein-expressing clones before sufficient quantities of highly purified material become available.

The ability of gel-purified OspC to induce mitogenesis of B cells from naive mice, however, was clearly demonstrated by lymphocyte proliferation and fluorescent cytometric assays. The fact that gel-purified flagellin caused little mitogenesis indicates that sample preparation made no significant contribution to the mitogenic effect of OspC. It is not known whether OspC, or other lipoproteins such as OspA and OspB, is involved in the pathogenesis of Lyme disease. However, peripheral blood lymphocytes from Lyme disease patients and healthy controls have been shown to mount similar proliferative responses to the spirochete (15). Increased B-cell activation in Lyme disease patients has been shown to correlate with the severity of disease as well (16). It is possible that outer surface lipoproteins are responsible for these effects. Such lipoproteins might stimulate autoreactive B cells that are otherwise anergic and initiate tissue injury in certain individuals. This type of autoimmune disease is usually associated with systemic rather than organ-specific complications, correlating with the manifestations of Lyme disease, which is a multisystemic disorder (17, 18). For this reason, the mitogenic capabilities of OspA, OspB, and OspC should be assessed in man, especially if these lipoproteins are components of a candidate vaccine.

William M. Whitmire's present address: 364 SW 62nd Blvd, #12, Gainesville, FL 32607.

Reprint requests: Claude P. Garon, Ph.D., Department of Health and Human Services, Laboratory of Vectors and Pathogens, Rocky Mountain Laboratories, Hamilton Montana 59840.

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