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Specific and Nonspecific Responses of Murine B Cells to Membrane Blebs of *Borrelia burgdorferi*

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Lymphocyte blastogenesis assays and immunoblotting were used to investigate and compare murine B-cell responses to preparations of extracellular membrane blebs (BAg) and spirochetes (Ag) of *Borrelia burgdorferi*. Immunoblotting BAg, Ag, and medium control preparations with serum from naive and infected C57BL/10 mice revealed that BAg and Ag had similar specific reactivity profiles except that major antigens of 83, 60, and 41 kDa were detected in Ag but not in BAg. It was determined that 1 μg (dry weight) of Ag contained 0.0051 and 0.0063 μg of outer surface proteins A (OspA) and OspB, respectively, whereas 1 μg (dry weight) of BAg contained 0.0024 μg of OspA and 0.0015 μg of OspB. Both BAg and Ag caused blastogenesis in cultures of spleen cells from both groups of mice, but BAg-stimulated lymphocytes exhibited significantly greater ($P \leq 0.05$) blastogenesis after 2 or 6 days of culture than did lymphocytes stimulated by Ag or medium control. Flow cytometry and antibody capture enzyme-linked immunosorbent assays identified responding lymphocytes as B cells which secreted polyclonal immunoglobulin M (IgM) but not IgG or IgA. Treatment of BAg and lipopolysaccharide controls with polymyxin B resulted in as much as 20.7 and 54.3% mean decreases in blastogenesis, respectively. Fractionation of BAg or Ag by ultracentrifugation before culture with spleen cells from naive mice indicated that B-cell blastogenesis was probably associated with spirochetal membranes. The results of this study demonstrate that specific humoral responses are directed towards extracellular membrane blebs which lack the 83-, 60-, and 41-kDa antigens of intact spirochetes and that blebs also possess significant nonspecific mitogenic activity for murine B cells. This activity was not due entirely to typical lipopolysaccharide or OspA and OspB lipoproteins.

Since *Borrelia burgdorferi* was first reported to be the etiologic agent of Lyme disease in 1982 (10), substantial efforts have been made to characterize host immune responses towards the spirochete (see reference 36 for a review). For example, antigen-specific T-cell responses have been demonstrated in infected hosts, and several investigators have been successful in cloning reactive T cells from Lyme disease patients (13, 28, 30, 40). Analysis of cytokine production by these T-cell clones indicated that they were of the T-helper type 1 cell lineage (40). Furthermore, infection generally results in production of specific immunoglobulins. This has led to the identification of several spirochetal immunogens and to the use of immunoglobulin-based assays for diagnostic tests (10, 11, 13, 34).

Nonspecific host responses to infection by the spirochete may also be important factors in Lyme disease. Habicht et al. (20) reported on the ability of *B. burgdorferi* to induce interleukin-1 production by cultured human and murine mononuclear phagocytes. A similar study has shown that tumor necrosis factor alpha is elaborated in vitro by human and murine adherent cells upon exposure to the spirochete as well (15). Elevated levels of interleukin-1 and tumor necrosis factor alpha were found in the synovial fluid of Lyme disease patients (4, 15). Since interleukin-1 and tumor necrosis factor alpha function as mediators of inflammation, these cytokines might contribute to the symptoms of Lyme disease. Additionally, nonspecific mitogenesis of B cells in patients with Lyme disease has been reported (32). Beck et al. (6) described a lipopolysaccharide (LPS) from the Lyme spirochete which could induce polyclonal B-cell activation, but other workers failed to confirm the presence of lipid A in *B. burgdorferi* (37). These data suggested that the spirochete is devoid of the type of LPS that is typically associated with gram-negative bacteria (37). Recently, sonicated preparations of *B. burgdorferi* were reported to cause mitogenesis of B cells from naive mice even after exposure of the preparations to polymyxin B (29). This observation also indicates that typical LPS is not responsible for B-cell mitogenesis in these preparations (29).

To understand immunity to Lyme disease, we became interested in host responses to extracellular membrane-bound vesicles of *B. burgdorferi*. Extracellular membrane-bound vesicles, or blebs, are spirochetal structures which are shed from the surface of the spirochete (19). Blebs have been reported to possess an 83-kDa multiprotein complex, and polyclonal rabbit antiserum raised against blebs or the multiprotein complex was capable of capturing sequestered spirochetal antigens from host tissues (17, 18). Sequestered antigens might stimulate protective, autoreactive, or nonspecific immune responses and therefore play a role in the pathogenesis of the disease. In the present study, we compare specific and nonspecific B-cell responses to blebs and whole-spirochete sonicates of *B. burgdorferi* in the murine model, demonstrate that bleb-induced mitogenesis is significantly greater than that caused by whole spirochetes, and suggest that B-cell mitogenesis is associated with spirochetal membranes with little typical LPS.

MATERIALS AND METHODS

Bacteria and antigen preparations. Low-passage (P6 to P8) strain Sh-2-82 of *B. burgdorferi*, which originated from adult *Ixodes dammini* ticks (Shelter Island, N.Y.), was grown in

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500-ml bottles of BSK II culture medium (2) at 34°C until a slight acid color change of the medium was evident. Spirochetes and membrane blebs were obtained from these cultures as described elsewhere (19). Briefly, spirochetes were isolated from the medium after centrifugation at 10,400 × *g* for 30 min at 25°C. These organisms were then washed and resuspended in 0.15 M phosphate-buffered saline (PBS [pH 7.2]; made with pyrogen-reduced Milli Q water [<1 EU/ml]; Millipore Corp., Bedford, Mass.). The supernatant was subjected to additional centrifugation at 20,200 × *g* for 15 min at 25°C and passaged through a 0.22-μm filter (Filter System 25942; Corning Laboratory Sciences, Corning, N.Y.). Subsequently, membrane blebs were pelleted from filtered medium by centrifugation at 235,000 × *g* for 90 min at 25°C and resuspended in PBS.

Antigen preparations of spirochetes or membrane blebs were produced by subjecting the suspensions described above to three cycles of freeze-thawing at −80°C and sonication for six 15-s cycles at a setting of 4 with a Branson sonicator (VWR Scientific, Inc., Seattle, Wash.) to disrupt intact bacterial structures. The resulting sonicates were centrifuged at 12,100 × *g* for 20 min at 4°C. Supernatants from spirochete (Ag) or bleb (BAg) sonicates were retained, filter sterilized, assayed for total protein (BCA Protein Assay Reagent; Pierce, Rockfield, Ill.) or dry weight, and frozen at −80°C. Equal volumes of uninoculated BSK II medium that were processed similarly to BAg served as a medium control (MC). Portions of MC, Ag, and BAg preparations were centrifuged at 100,000 × *g* for 2 h at 4°C, and the resulting supernatants were separated from pelleted material. Pelleted material from the respective preparations was brought back to the original volume in PBS, and all fractionated (supernatants and pellet material) preparations were subsequently frozen at −80°C.

Experimental animals and serum production. Female C57BL/10 mice were obtained from a colony at Rocky Mountain Laboratories, Hamilton, Mont., and used at 4 to 8 weeks of age. Infected animals were inoculated subcutaneously with 10^7 low-passage, viable *B. burgdorferi* Sh-2-82 organisms in 0.05 ml of PBS 2 to 3 weeks prior to experimentation. Immune serum was processed from the pooled blood of subcutaneously infected animals that had been exsanguinated by axillary bleed 1 month after inoculation. Normal serum from age-matched mice that were sham-inoculated with PBS was processed in a similar fashion, and both types of sera were frozen at −20°C until needed. Immune and normal sera were assessed for spirochete-specific immunoglobulin by an indirect immunofluorescence assay with acetone-fixed spirochetes and fluorescein-conjugated goat anti-mouse immunoglobulin G (IgG; heavy- and light-chain specific; Boehringer Mannheim Biochemicals, Indianapolis, Ind.).

LBA. For the lymphocyte blastogenesis assay (LBA), pooled cell suspensions were prepared from the spleens of three age-matched naive or infected mice by gentle dissociation with sterile scissors and syringes. Erythrocytes were removed by hypotonic lysis, and the resulting cell suspensions were washed and resuspended at a concentration of 2 × 10^6 viable cells per ml in RPMI 1640 culture medium (made with pyrogen-reduced water; GIBCO, Grand Island, N.Y.) supplemented with 20 mM glutamine and 200 U of penicillin per ml. Sextuplet or triplicate cultures of both groups of spleen cells 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 with 20% (vol/vol) fetal bovine serum (Hyclone Laboratories Inc., Logan, Utah) and either mitogen (50 μg of LPS from *Escherichia coli* O111:B4 per ml; Difco Laboratories, Detroit, Mich.), PBS, or dilutions of MC, Ag, or BAg. After 1 or 5 days of incubation at 37°C in a humidified 95% air–5% CO_2 atmosphere, 0.5 μCi of [methyl-^{3}H]thymidine (specific activity, 6.7 Ci/mmol; NEN Research Products, Du Pont Co., Wilmington, Del.) in 50 μl of RPMI was added to each culture. Cultures were incubated for an additional 18 h, harvested with a PHD cell harvester (Cambridge Technology, Inc., Cambridge, Mass.), and incorporation of [^{3}H]thymidine was determined by liquid scintillation counting. The stimulation index (SI) was calculated by dividing the counts per minute (cpm) in stimulated (LPS, MC, Ag, or BAg) cultures by the mean cpm in sextuplet unstimulated (control) cultures. Results are shown as the mean SI of triplicate spleen cell cultures from each group of animals ± standard error of the mean (SEM). The results were analyzed statistically by Student's *t* test or single-factor analysis of variance (28). The level of significant difference was at $P \leq 0.05$ in all cases.

Fluorescence-activated cytometric analysis. After 7 days of exposure to BAg (50 μg [dry weight] per well), spleen cell cultures from naive mice were removed from an LBA plate and placed into round-bottomed wells of a 96-well microtiter plate (Flow Laboratories). Following centrifugation at 300 × *g* for 10 min, cultures were 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% [vol/vol] fetal bovine serum and 10 mM sodium azide) and incubated for 20 min on ice. Cultures were then washed twice, resuspended 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, Calif.). Fresh unstimulated spleen cells obtained from a naive mouse were treated with a monoclonal antibody against murine CD4 (ImmunoSelect; Life Technologies, Inc., Gaithersburg, Md.) to set limits (boxed area) for detection of blasting cells.

Detection of in vitro murine immunoglobulin. Supernatant fluid from cultures of spleen cells from naive animals that had been exposed to MC, LPS, or BAg for 6 days in the LBA was tested for the presence of class, subclass, and light-chain types of murine immunoglobulin by enzyme immunoassay (mouse-hybridoma subtyping kit; Boehringer Mannheim). The assay was performed according to the manufacturer's instructions in a 96-well flat-bottomed microtiter plate (Flow), and the A_{405} was determined with a Titertek Multiscan Plus MKII (Flow) plate reader. Culture supernatants with an A_{405} of ≤0.05 were considered negative for heavy or light chains of murine immunoglobulin.

Polymyxin B treatment. Preparations of RPMI (unstimulated control), BAg, or LPS were treated with 10, 20, or 40 μg of polymyxin B sulfate (Sigma Chemical Co., St. Louis, Mo.) per ml for 20 min at room temperature. Polymyxin B-treated and untreated RPMI, BAg, and LPS were then used to stimulate spleen cells from naive mice for 2 days in order to determine the effect of polymyxin B treatment on lymphocyte blastogenesis. The mean SI was calculated for triplicate cultures of each preparation. Results are given as mean percent decrease in blastogenesis ± SEM for polymyxin B-treated preparations compared with corresponding untreated (control) preparations.

Polyacrylamide gel electrophoresis. MC, Ag, or BAg preparations were diluted 1:2 in 2× sodium dodecyl sulfate (SDS) solubilizing solution (4% SDS, 0.5 M Tris [pH 6.8], and 20% glycerol with 10% 2-mercaptoethanol) and heated at 95°C for

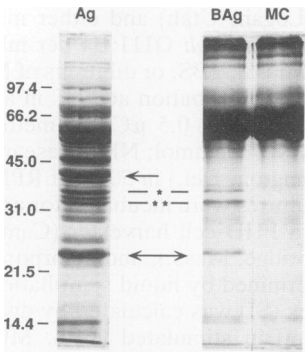

FIG. 1. SDS-polyacrylamide gel electrophoresis of reduced Ag, BAg, and MC in a 12% polyacrylamide gel stained with Coomassie brilliant blue. Each lane contains 25 μg of total protein from the indicated samples. All lanes were scanned by laser densitometry. The positions of the 41-kDa (single arrow), OspB (*), OspA (**), and 23-kDa (double arrow) protein bands are indicated. Positions of molecular size standards are indicated on the left (in kilodaltons).

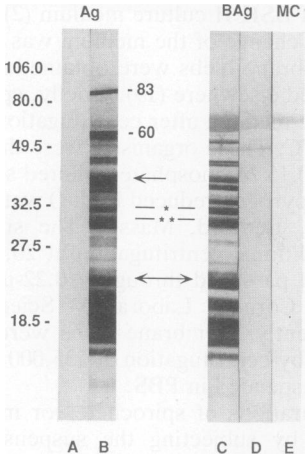

FIG. 2. Immunoblots of reduced Ag, BAg, and MC (transferred from an SDS–12% polyacrylamide gel) reacted with normal (lanes A and D) and immune (lanes B, C, and E) serum. The positions of the 41-kDa (single arrow), OspB (*), OspA (**), and 23-kDa (double arrow) proteins are indicated. Note the specific reactivity to major antigens of 83, 60, and 41 kDa in Ag (lane B) but not in BAg (lane C). Positions of molecular size standards are indicated on the left (in kilodaltons).

5 min. Solubilized preparations as well as pre- or unstained molecular size standards (Bio-Rad Laboratories, Richmond, Calif.) were subjected to SDS-polyacrylamide gel electrophoresis in 12% polyacrylamide slab gels with a Mini Protean II gel apparatus (Bio-Rad) and the discontinuous buffer system described by Laemmli (24). Following electrophoresis at 200 V for approximately 50 min, gels were either stained with Coomassie brilliant blue and analyzed by laser densitometry (Ultroscan XL Enhanced Laser Densitometer; LKB Produkter AB, Bromma, Sweden) or immunoblotted.

Immunoblotting of spirochetal antigens. Reduced MC, Ag, and BAg preparations were electrophoretically transferred to nitrocellulose sheets (0.1-μm pore size; Schleicher & Schuell, Inc., Keene, N.H.) in a Mini Trans-Blot Cell (Bio-Rad) for 1.5 h at 100 V (38). Following transfer, nitrocellulose sheets were incubated in PBS with 0.05% Tween 20 overnight at 4°C to block nonspecific binding sites. Nitrocellulose sheets were then cut into 4-mm-wide strips and reacted with murine normal or immune serum (indirect immunofluorescence assay titers of 0 and 1:1,280, respectively) diluted 1:100 in blocking buffer for 2 h at room temperature under constant agitation. After exposure to horseradish peroxidase-conjugated goat anti-mouse IgG (heavy- and light-chain specific; Pierce), diluted 1:2,500 in blocking buffer for 1 h at room temperature, bound peroxidase activity was detected with peroxidase substrate solution (22). Nitrocellulose strips were also reacted with monoclonal antibodies 5332 (anti-outer surface protein A [OspA]), 5TS (anti-OspB), and H9724 (anti-41-kDa protein [flagellin]). These monoclonal antibodies were generously supplied by Tom Schwan (Rocky Mountain Laboratories).

RESULTS

Analysis of Ag, BAg, and MC preparations by SDS-polyacrylamide gel electrophoresis in conjunction with Coomassie brilliant blue stain revealed comparatively fewer protein bands in BAg than in Ag (Fig. 1). A protein band that corresponds to flagellin was detected in Ag but not BAg (Fig. 1, single arrow). However, OspB and OspA were present in both preparations (Fig. 1, single and double asterisks, respectively), as was a protein band of 23 kDa (Fig. 1, double arrow). The identities of OspA, OspB, and flagellin were confirmed by immunoblots with monoclonal antibodies 5332, 5TS, and H9724 (data not shown). Scanning of these protein profiles by laser densitometry revealed that OspA and OspB constituted 5.1 and 6.3%, respectively, of the Ag profile but only 2.4 and 1.5%, respectively, of the BAg profile (Fig. 1). Since 10 μg (dry weight) of Ag or BAg was found to contain 1 μg of total protein, 1 μg (dry weight) of Ag was calculated to contain 0.0051 μg of OspA and 0.0063 μg of OspB, and 1 μg (dry weight) of BAg was determined to contain 0.0024 and 0.0015 μg of OspA and OspB, respectively. None of the aforementioned protein bands were present in the MC preparation, but several bands comigrated in BAg and MC, especially in the high-molecular-weight region of the gel (Fig. 1). The most prominent high-molecular-weight band in BAg and MC represents bovine serum albumin, which was a component of BSK II medium (Fig. 1). Further scanning by laser densitometry demonstrated that bovine serum albumin constituted 66 and 80% of the protein profiles of BAg and MC, respectively.

Immunoblots of Ag, BAg, and MC preparations that were reacted with normal and immune serum are shown in Fig. 2. The spirochete-specific antibody reactivities to antigens of Ag and BAg were remarkably similar and included reactivity to OspB and OspA (single and double asterisks, respectively), a 23-kDa protein (double arrow), and others that were present in both preparations (Fig. 2). Reactivity with spirochete-specific antibody to flagellin (single arrow) and major antigens of approximately 83 and 60 kDa were found with Ag alone (Fig. 2). Monoclonal antibody H9724 against flagellin reacted to immunoblots of Ag but not BAg (data not shown). Normal serum did not react to immunoblots of Ag, and the reactivity of normal serum to BAg was minimal, with only a small degree of nonspecificity being evident in the high-molecular-weight region of the immunoblot (Fig. 2). The reactivity of murine immune serum was confined to a single band in the high-molecular-weight nonspecific region on immunoblots of MC (Fig. 2).

Lymphocytes in spleen cultures from naive and infected mice demonstrated significant blastogenic responses to Ag and BAg in the LBA at 6 days after culture initiation (Fig. 3).

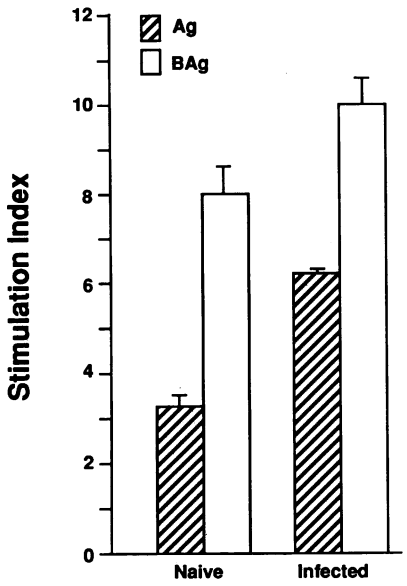

FIG. 3. Blastogenic responses (SI ± SEM) to Ag and BAg by lymphocytes in spleen cell cultures from naive and *B. burgdorferi*-infected mice 6 days after culture initiation. Concentrations of Ag and BAg were 100 and 50 μg (dry weight) per well, respectively. The mean values ± SEM for control spleen cell cultures from naive and infected mice were 593.3 ± 41.8 and 319.7 ± 13.7 cpm, respectively.

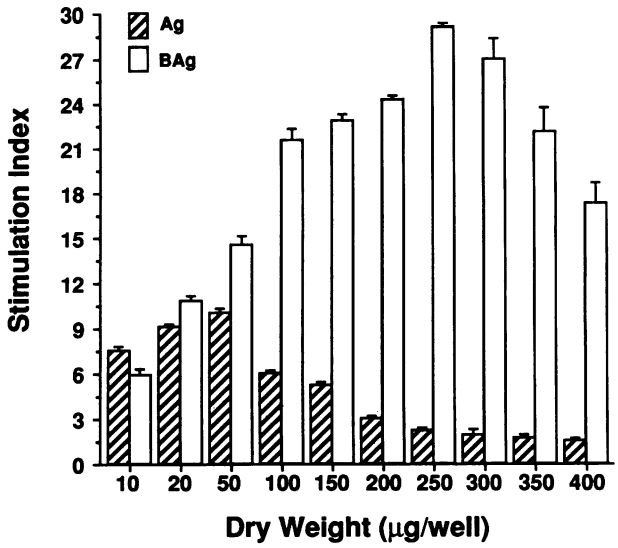

FIG. 4. Blastogenic responses (SI ± SEM) of spleen cell cultures from naive mice over several concentrations of Ag and BAg 2 days after culture initiation. Note the significantly greater ($P \leq 0.05$) response to BAg at 2 days than at 6 days (Fig. 3) after culture initiation with spleen cells from naive mice. The mean value ± SEM for control spleen cell cultures after 2 days was 258.0 ± 24.4 cpm.

However, exposure of lymphocytes from either group of animals to BAg resulted in a significantly greater response than did exposure to Ag, even though the concentration (dry weight) of Ag (100 μg per well) was twice as much as that of BAg (50 μg per well; Fig. 3).

Time course experiments over 2-day intervals beginning 2 days after culture initiation of spleen cells from naive mice revealed that blastogenic responses to Ag or BAg after 2 days were similar to or greater than blastogenic responses at 4 or 6 days (data not shown). Figure 4 illustrates the results of the LBA with spleen cells from naive mice following exposure to several concentrations of Ag or BAg for 2 days. Blastogenesis by these lymphocytes demonstrated a typical concentration-dependent response. However, the responses elicited by BAg became significantly greater than those by Ag at 100 μg per well and remained greater with increasing concentrations of either preparation (Fig. 4). The mean SIs for MC at concentrations of 100 and 200 μg per well were 2.6 ± 0.5 and 2.9 ± 0.1, respectively.

Preparations of Ag and BAg that were fractionated by ultracentrifugation were capable of inducing spleen lymphocytes from naive mice to undergo blastogenesis. However, the blastogenic responses caused by pellet fractions of Ag and BAg after 2 days of culture were significantly greater than those caused by the supernatant fractions of the respective preparations (Table 1). There were no significant differences in mean SI between any fractionated or unfractionated MC preparations or between Ag or BAg pellet fractions and their corresponding unfractionated counterparts (Table 1).

The effects of polymyxin B treatment on LPS and BAg preparations following 2 days of culture in the LBA are shown in Table 2. Three different concentrations of polymyxin B resulted in 17.3% ± 0.7% or 20.7% ± 3.8% mean decrease in blastogenesis of BAg-stimulated lymphocytes, whereas identical concentrations of polymyxin B caused as much as a 54.3% ± 4.9% mean decrease in the blastogenic response of lymphocytes stimulated by LPS (Table 2). Polymyxin B had no effect on unstimulated control cultures.

Treatment of BAg-stimulated spleen lymphocytes with monoclonal antibodies directed against Thy-1.2 or B220 (murine pan-T-cell and mature and immature murine B-cell markers, respectively) in conjunction with fluorescence-activated cytometric analysis revealed that responding lymphocytes were of the B-cell lineage (Fig. 5). Figure 5A illustrates resting populations of high-fluorescing $CD4^+$ T cells (paired arrows) and other low-fluorescing cells (single arrow) in a fresh murine spleen cell sample (i.e., both populations of cells lie to the left of the boxed area). Treatment of BAg-stimulated lymphocytes with anti-Thy-1.2 demonstrated that the majority of T cells remained in a resting state, but the presence of blasting cells was detected in the low-fluorescing population, as a large proportion of these cells were located in the boxed area (single arrow, Fig. 5B). Further analysis indicated that blasting cells reacted with fluorescein-conjugated anti-B220 (paired arrows, Fig. 5C).

Analysis of culture supernatants by enzyme immunoassay

TABLE 1. Murine lymphocyte blastogenesis after stimulation by unfractionated or fractionated spirochetal and control preparations

Prepn[a]	Mean SI ± SEM (n = 3)		
	Supernatant	Pellet	Unfractionated
Ag	6.3 ± 0.1[b]	15.2 ± 1.1	12.7 ± 0.2
BAg	7.5 ± 0.3[b]	19.5 ± 0.8	23.0 ± 1.2
MC	0.8 ± 0.0	1.6 ± 0.1	1.8 ± 0.2

[a] The concentrations of unfractionated Ag, BAg, and MC preparations were 50, 150, and 150 μg (dry weight) per well, respectively. These concentrations of Ag and BAg were previously determined to generate nearly optimal blastogenic activity (see Fig. 4).

[b] Significant difference ($P \leq 0.05$) from corresponding pellet and unfractionated preparations. The mean ± SEM for control spleen cell cultures was 471.3 ± 102.2 cpm.

TABLE 2. Effect of polymyxin B treatment of BAg and LPS on murine lymphocyte blastogenesis[a]

Prepn	Polymyxin B (μg/ml)	Mean % decrease in blastogenesis ± SEM
BAg	10	20.7 ± 3.3
	20	17.3 ± 0.7
	40	20.7 ± 3.8
LPS	10	40.3 ± 1.4
	20	42.7 ± 1.8
	40	54.3 ± 4.9

[a] BAg (100 μg [dry weight] per well) or LPS (50 μg/ml) preparations were treated with the indicated concentrations of polymyxin B for 20 min at room temperature. Blastogenesis was assessed 2 days after culture initiation of spleen cells from naive animals, and the mean percent decrease in blastogenesis of triplicate cultures ± SEM was calculated for treated versus untreated (control) BAg or LPS preparations. Mean percent decrease in blastogenesis of treated BAg preparations was significantly less ($P \leq 0.05$) than that of treated LPS preparations at all concentrations of polymyxin B tested.

indicated that only IgM with kappa and lambda light chains was produced by BAg- or LPS-stimulated spleen B cells from naive mice. The MC did not cause elaboration of immunoglobulin from spleen cell cultures. Absorbance units for IgM, kappa, or lambda light chains, respectively, in culture supernatants after stimulation by the indicated preparations were as follows: LPS, 0.587, 0.658, and 1.251; and BAg, 0.314, 0.317, and 0.624.

DISCUSSION

The pathogenesis of Lyme disease appears to be a complicated process, and it has been suggested that nonspecific as well as specific host cell responses may account for the tissue injury that is observed in Lyme disease patients (31, 36). In recent reports, sonicated or whole spirochetes of *B. burgdorferi* were shown to cause nonspecific, in vitro mitogenesis of B cells from several strains of mice, including endotoxin-sensitive C3H/HeN and endotoxin-resistant (LPS-nonresponsive) C3H/HeJ mice (16, 29). Furthermore, it was shown that polymyxin B had little effect on mitogenesis caused by sonicated spirochetes (29). Polymyxin B binds to the lipid A portion of typical LPS and decreases its ability to cause blastogenesis (26). These data suggest that typical bacterial LPS was not responsible for the mitogenic activity caused by sonicated or whole spirochetes (16, 29).

In the present study, our original intent was to examine antigen-specific responses to the spirochete by lymphocytes from infected mice. However, we found that exposure to BAg or Ag results in significant blastogenesis by spleen lymphocytes from naive and infected mice after 6 days of culture. This indicates that the blastogenic response that was induced by these preparations did not require prior immunization and was therefore mitogenic in nature (7). The fact that blastogenic responses by spleen lymphocytes from naive mice were maximal following 2 days of culture further demonstrates the mitogenic effects of BAg and Ag (7). Differences in the number of B cells in spleen cell preparations may be the reason for different levels of BAg- or Ag-induced blastogenesis between naive and infected mice. These results, along with fluorescence-activated cytometric analysis of BAg-stimulated spleen cells from naive animals, not only confirm the reported ability of sonicated spirochetes to cause mitogenesis of murine B cells (29), but demonstrate that greater B-cell mitogenic activity resides in BAg than in

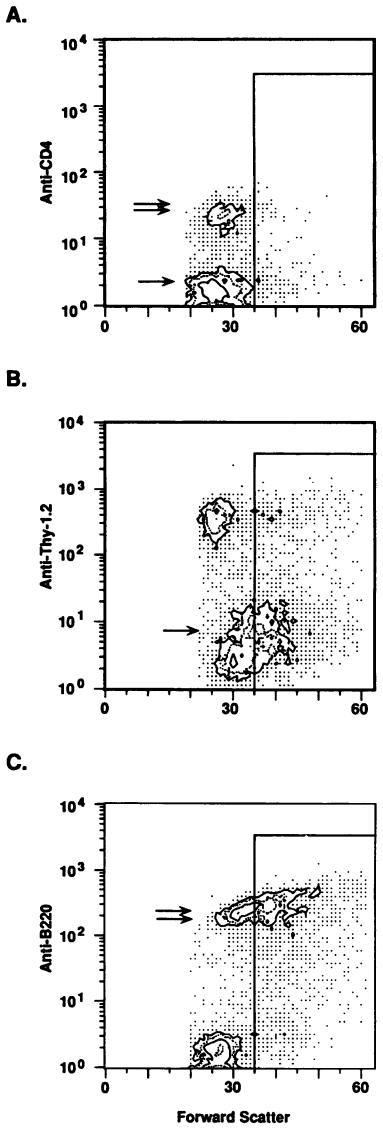

FIG. 5. Fluorescence-activated cytometric analysis of fresh unstimulated control (A) and BAg-stimulated (7 days after culture initiation; B and C) spleen cells from naive mice with anti-CD4 (A), anti-Thy-1.2 (B), or anti-B220 (C) monoclonal antibodies. Single and paired arrows indicate low- and high-fluorescing cell populations, respectively, whereas data points in the boxed areas represent blasting (increased cell size) cells. Note that T cells present in BAg-stimulated cultures are predominately small, similar to $CD4^+$ T cells in the fresh control sample, while the $B220^+$ B cells include many large blast cells after stimulation.

spirochete sonicates of *B. burgdorferi*. Since the PBS and RPMI solutions were made with pyrogen-reduced water, it is unlikely that endotoxin contamination was responsible for the mitogenic activity that was induced by Ag or BAg. Calculation of the SI also cancels out any mitogenic activity that might be present in RPMI alone. The components of BSK II medium, such as bovine serum albumin, did not appear to contribute to the mitogenic activity of BAg, because MC stimulation of spleen lymphocytes from naive or infected mice remained low and was significantly less than that caused by BAg.

Examination by LBA over increasing concentrations of

Ag or BAg revealed significant differences in the mitogenic activity that was induced by these preparations. Since BAg consists of membrane-bound vesicles which are shed from the surface of the spirochete, it is possible that BAg contains more membrane-associated mitogenic factors than Ag at equal concentrations. On the other hand, cytotoxic or inhibitory substances may be present in Ag that prevent its optimal mitogenic activity, especially since Ag-induced mitogenesis peaked at relatively low concentrations.

In an attempt to determine the localization of mitogenic activity, BAg and Ag preparations were fractionated into pellet and supernatant forms by ultracentrifugation. Since the majority of the mitogenic activity remained with pellet fractions of BAg as well as Ag, it is likely that mitogenic activity is associated with the membranes of the spirochete. Outer membrane proteins OspA and OspB have been shown to exist intracellularly, and recombinant forms of these lipoproteins are capable of eliciting mitogenesis of murine B cells (8, 9, 16). Since Ag and BAg contain both OspA and OspB, it is possible that these proteins contribute to the mitogenic activity that is exhibited by either Ag or BAg or their fractionated forms. However, much less OspA plus OspB was present in BAg than in Ag at equal concentrations, but significantly greater B-cell mitogenesis was induced by BAg. Therefore, it seems likely that other potent mitogenic factors are present in *B. burgdorferi*. Such mitogenic factors may be nonproteinaceous in nature. Experiments are under way to identify and characterize other spirochetal mitogens.

Treatment of BAg and LPS with polymyxin B demonstrated that BAg-induced mitogenesis was not due entirely to typical LPS. Beck et al. (6) described the extraction of LPS from the Lyme disease spirochete. Conversely, Takayama et al. (37) failed to identify lipid A in spirochete preparations after using two different extraction methods. Extraction techniques for LPS were not used in the present study. It is possible that BAg contains a mixture of LPS and non-LPS mitogens, which may account for its partial inhibition by polymyxin B. However, our results concerning non-LPS mitogenic factors contained within BAg agree with recent findings by Schoenfeld et al. (29) and de Souza et al. (16), which describe non-LPS mitogenic activity by whole-spirochete sonicates and whole spirochetes, respectively. Thus, it appears that *B. burgdorferi* is lacking significant amounts of typical gram-negative bacterial LPS.

Peptidoglycan extracted from *B. burgdorferi* has been shown to have a mitogenic effect on murine splenocytes, but 25 μg/ml was necessary to achieve an effect that was greater than twice background levels (5). Since Ag- or BAg-induced mitogenesis was at least five times greater than background at the lowest concentrations (10 μg [dry weight] per well) used in the present study and peptidoglycan only makes up 0.01% (dry weight) of the spirochete (5), it is unlikely that peptidoglycan alone was responsible for this mitogenic activity. Although it is not known whether peptidoglycan is associated with blebs that are shed from the surface of the spirochete, it is possible that peptidoglycan contributed to the mitogenic effect caused by the highest concentrations of BAg. Chemical analysis of BAg will be necessary to answer this question.

Besides OspA and OspB, only one other major protein band of 23 kDa is common to Ag and BAg following staining of SDS-polyacrylamide gels with Coomassie brilliant blue. This 23-kDa band is probably the major pC antigen, which has been reported to range in size from approximately 21 to 23 kDa and to react with murine, rabbit, and human antisera (39). This protein also appears to be associated with the outer membrane of the spirochete (23).

The reactivity patterns displayed by murine immune serum on immunoblots of Ag and BAg were remarkably similar and demonstrate specific B-cell responses to BAg. However, major antigens of 83, 60, and 41 kDa in Ag were absent in BAg. Antibodies (IgG and IgM) from infected individuals have been found to react to an 83-kDa antigen of solubilized spirochetes, and a chromosomal gene for this antigen has been cloned and expressed as well (9, 20). Further characterization of the 83-kDa antigen revealed that the actual size of the antigen was 79.8 kDa, based on amino acid sequencing (27). The 83-kDa antigen described in the present study is most likely the same as the previously described 79.8-kDa antigen. The 60-kDa antigen has been described as an immunodominant antigen that is found in remotely related bacteria (21). The fact that the 83- and 60-kDa antigens are missing in BAg may indicate that these antigens are not associated with spirochetal membranes. Similarly, reactivity to the flagellin band at 41 kDa (3) would not be expected in BAg, since endoflagella would remain with intact spirochetes. The lack of flagellin in BAg is consistent with results described by Dorward et al. (18). In any event, the 83-, 60-, and 41-kDa antigens serve as negative markers for BAg, and lack of the 41-kDa antigen is evidence for the absence of whole spirochetes in BAg. Other immunoreactive antigens that are common to Ag and BAg include OspA, OspB, and the 23-kDa antigen. The immune serum raised in the present study did not react with the previously described 83-kDa multiprotein complex of blebs on immunoblots (18).

The ability of BAg to cause production of IgM with both light chains (polyclonal) is consistent with a B-cell mitogenic response that is T-cell independent (1). Similar in vitro generation of IgM from murine B cells stimulated with sonicated spirochetes, whole spirochetes, or recombinant OspA and OspB has been described in previous studies as well (16, 29). However the generation of IgG from stimulated B-cells is also described in one of these reports (16). Although the subclass of IgG was not distinguished in that study (16), T-cell-independent B-cell mitogens such as LPS are known to cause secretion of IgG3 in addition to IgM from murine B cells but not switching to other isotypes of immunoglobulin (1). Differences in the strain of mice or preparations used in the present study may account for the lack of IgG secretion described in the previous report.

Since antigens contained within spirochetal blebs have been observed in the tissues of infected hosts (18), and specific as well as nonspecific host responses are directed towards these blebs, it is interesting to speculate that blebs may be associated with the pathogenesis of Lyme disease. For example, it has been suggested that elevated total IgM levels correlate with neurologic, cardiac, or joint involvement (35). Furthermore, increased levels of IgM in Lyme disease patients has been shown to be a result of B-cell hyperactivity, which is caused by both antigenic and mitogenic stimulation of B cells (32). Some patients possess serum agglutinins which agglutinate heterologous erythrocytes as well (32). It is conceivable that spirochetal blebs in host tissues play a role in these phenomena, which may result in the production of immune complexes or autoreactive immunoglobulins. Whether blebs of *B. burgdorferi* possess mitogenic activity for human B cells as they do for murine B cells remains to be determined.

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