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Interleukin-6 Enhances Production of Anti-OspC Immunoglobulin G2b Borreliacidal Antibody

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Protection against infection with *Borrelia burgdorferi* is dependent primarily on induction of complement-dependent antibody that can kill the spirochete. Measuring the production of sustained high levels of borreliacidal antibody is thus paramount for determining potential vaccine efficacy. We investigated the borreliacidal antibody response in sera and the amount of antibody produced by cultured lymph node cells of C3H/HeJ mice vaccinated with outer surface protein C (OspC). We showed that recombinant OspC was a weak stimulant of borreliacidal antibody production compared to whole cells of OspC-expressing *B. burgdorferi*. Mice vaccinated with *B. burgdorferi* in adjuvant produced a high level (titer, 5,120) of anti-OspC borreliacidal antibody, which waned rapidly. Similarly, borreliacidal antibody production by cultured lymph node cells from vaccinated mice peaked soon after vaccination and then decreased. Treatment of lymph node cells with interleukin-6 (IL-6) augmented borreliacidal antibody production, particularly immunoglobulin G2b, whereas treatment with anti-IL-6 inhibited the borreliacidal response. These findings demonstrate a previously unrecognized role for IL-6 in borreliacidal antibody production that may have important implications for vaccine development.

The increased prevalence of and morbidity associated with Lyme borreliosis (6) has prompted the development of vaccines to prevent infection with *Borrelia burgdorferi*. The leading vaccine candidate has been outer surface protein A (OspA) (10, 20, 25), primarily because the majority of *B. burgdorferi* isolates express significant concentrations of this antigen (9), which induces protective borreliacidal antibody (20). In addition, several investigators have demonstrated that OspA is present predominantly on *B. burgdorferi* organisms residing in the midguts of infected *Ixodes* ticks (7, 28). Two Lyme borreliosis recombinant Osp A (rOspA) vaccines have been shown to be protective in human clinical trials (29, 30). Protection, however, is dependent on the production of sufficient levels of anti-OspA borreliacidal antibody (23) to destroy spriochetes in the midguts of infected ticks (11).

Previously, Schwan et al. (28) demonstrated that *B. burgdorferi* down-regulated the expression of OspA shortly after an infected tick attached to its mammalian hosts. Down-regulation of OspA hinders the ability of vaccination with OspA to provide protection since Lyme borreliosis spirochetes could potentially establish infection even in the presence of borreliacidal anti-OspA antibody. Thus, if the level of protective antibody decreases far enough that it is below the level necessary to kill spirochetes in the midgut of ticks, a sufficient anamnestic borreliacidal anti-OspA antibody response might not occur before spirochetes down-regulate OspA (23). Having down-regulated OspA, the organisms could then establish infection in the host despite presence of borreliacidal anti-OspA antibody.

This finding has intensified the search for additional *B. burgdorferi* antigens that could be used to stimulate a protective borreliacidal antibody response. OspC has shown promise as an alternate vaccine candidate. OspC is rapidly synthesized by *B. burgdorferi* shortly after attachment of infected ticks to the host (28), and OspC-expressing spirochetes expand preferentially over those expressing OspA (21). Anti-OspC antibody is also among the first antibodies detected in patients and experimental animals with early Lyme borreliosis (8, 13). Moreover, several investigators have demonstrated that vaccination with OspC can provide protection against infection with *B. burgdorferi* (14, 15, 24, 25). Although heterogeneity of OspC exists among isolates of *B. burgdorferi* (12, 32), the epitopes responsible for the induction of borreliacidal antibody might be conserved. We (5, 27) detected borreliacidal anti-OspC antibody in sera from patients with early Lyme borreliosis from different geographic areas by using one isolate of *B. burgdorferi*.

In this study, we demonstrate that vaccination with rOspC or *B. burgdorferi* isolate 50772, which expresses high levels of OspC, induces borreliacidal anti-OspC antibody in vivo. However, the levels of borreliacidal anti-OspC antibody were not sustained. Similar borreliacidal antibody responses were obtained with immune lymph node cells, despite being cultured in vitro with *B. burgdorferi* expressing high levels of OspC. Addition of interleukin-6 (IL-6) overcame this effect by inducing significant increases in borreliacidal antibody production, particularly of the immunoglobulin G2b (IgG2b) subclass. Conversely, treatment with anti-IL-6 significantly decreased the

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production of anti-OspC borreliacidal antibody. These results suggest that a cytokine-induced mechanism may dampen the production of borreliacidal antibody in vivo despite the presence of *B. burgdorferi*. For vaccination with OspC to provide long-term protection, the inhibitory mechanism(s) of anti-OspC antibody production needs to be elucidated.

MATERIALS AND METHODS

Mice. Inbred C3H/HeJ male and female mice (8 to 12 weeks old) weighing 20 to 30 g were obtained from our breeding colony located at the Wisconsin State Laboratory of Hygiene and housed at four mice per cage. Food and water were provided ad libitum.

Organism. *B. burgdorferi* sensu stricto isolate 50772, originally isolated from an *Ixodes scapularis* tick, was obtained from John. F. Anderson (Connecticut Agricultural Experimental Station, New Haven, Conn.). The spirochete lacks the *ospA/B* operon and therefore does not express OspA or OspB but does express high levels of OspC (27). The original suspension of spirochetes was serially 10-fold diluted in Barbour-Stoenner-Kelly (BSK) medium, which is capable of supporting growth from a single organism. The resultant population of spirochetes was then passaged 10 times in fresh BSK medium at 35°C, dispensed as 200-μl samples into 1.5-ml screw cap tubes (Sarstedt, Newton, N.C.), and stored at −70°C until used.

Preparation of vaccines. *Escherichia coli* JM 109 (Promega, Madison, Wis.) containing the *ospC* gene was grown for 12 h at 37°C in 100 ml of 2× tryptone-yeast extract (TY) broth containing ampicillin. The culture was diluted 1:10 with 2× TY broth and incubated for an additional 1 h. Isopropyl-β-D-thiogalactopyranoside (final concentration, 0.1 mM; Sigma, St. Louis, Mo.) was added to the culture, which was then incubated for 4 h. The suspension was centrifuged at 10,000 × *g* for 15 min at 4°C, resuspended in purification buffer (50 mM Tris [pH 8.0], 50 mM NaCl, 2 mM EDTA, 0.1% Triton X-100), and lysed by sonication (model W 350 instrument; Branson Sonic Power, Danbury, Conn.). Lysed *E. coli* cells were centrifuged at 10,000 × *g* for 15 min, and the supernatant was passed over a column containing SoftLinks resin (Promega) at a rate of 0.5 ml/min at 4°C. The column was then eluted with 5 mM biotin (Sigma), and the recovered fractions were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Purified OspC (60 μg) was administered with 0.5 ml of 1% aluminum hydroxide (Reheis, Berkeley Heights, N.J.) or without adjuvant to vaccinate mice.

A whole-cell vaccine was also prepared. *B. burgdorferi* organisms were grown in 1 liter of BSK medium for 6 days, pelleted by centrifugation (10,000 × *g* at 15°C for 10 min), and washed three times with phosphate-buffered saline (PBS; pH 7.4). Samples (1 ml) containing 4.5×10^9 spirochetes/ ml were then stored at −70°C until used. Subsequently, a frozen suspension of *B. burgdorferi* was thawed, diluted in PBS, and mixed with or without a sufficient volume of 1% aluminum hydroxide to yield 4×10^6 spirochetes/ml.

Vaccination of mice. Mice were anesthetized with methoxyflurane contained in a mouth-and-nose cup and injected subcutaneously in the inguinal region with 0.25 ml (approximately 10^6 *B. burgdorferi* cells) of the vaccine preparations. The suspensions contained approximately 100 μg of borrelial protein. Other mice were injected subcutaneously in the inguinal region with 0.25 ml of OspC (30 μg) with or without aluminum hydroxide. Sham-vaccinated mice received either BSK medium or aluminum hydroxide alone.

Mouse sera. Sera were obtained from vaccinated mice anesthetized with methoxyflurane contained in a mouth-and-nose cup and bled by intracardiac puncture. The blood was allowed to clot, and serum was separated by centrifugation at 500 × *g*, divided into 0.25-ml samples, dispensed into 1.5-ml screw-cap tubes (Sarstedt), and frozen at −70°C until use. Equal volumes of thawed sera from the same group of mice were pooled before use.

Isolation of lymph node cells. Mice were euthanized by CO_2 inhalation at 0, 7, 14, 21, 28, 35, and 63 days after vaccination with preparations of *B. burgdorferi* or rOspC. Inguinal lymph nodes were removed from vaccinated and nonvaccinated mice and placed in cold Dulbecco's modified Eagle's medium (DMEM; Sigma). Single-cell suspensions of lymph node cells were prepared by teasing apart the lymph nodes with forceps and pressing them through a sterile stainless steel 60-mesh screen with a 5-ml syringe plunger into antimicrobial agent-free cold DMEM supplemented with 10% heat-inactivated fetal bovine serum (FBS), L-glutamine, and 2-mercaptoethanol. The lymph node cells were then washed twice with DMEM by centrifugation (500 × *g* at 4°C for 10 min). Supernatants were decanted, and pellets were resuspended in 1 ml of cold DMEM. Cell viability was assessed by trypan blue exclusion in a hemocytometer.

Production of antibody in vitro. Sterile six-well flat-bottom tissue culture dishes (Becton-Dickinson, Lincoln Park, N.J.) were inoculated with lymph node cells (10^7) obtained from vaccinated or nonvaccinated mice and 10^6 viable *B. burgdorferi* cells. DMEM was added to the suspensions of cells and *B. burgdorferi* to bring the volume to 2 ml. After 1, 3, 5, 7, and 9 days of cultivation at 37°C in the presence of 5% CO_2, a 1.0-ml sample of each supernatant from triplicate cultures was removed after gentle agitation and replaced with an equal volume of warm DMEM. In other experiments, rIL-6 (0.01 or 0.1 μg) or anti-IL-6 (1, 10, or 100 μg) (R & D Systems, Minneapolis, Minn.) was added to cultures of lymph node cells after 5 min of incubation. Control cultures for anti-IL-6 were also incubated with a goat isotype control. The supernatants were collected, centrifuged at 10,000 × *g* for 8 min to remove spirochetes and other cellular debris, and stored at −70°C until needed.

Detection of borreliacidal antibody by membrane filtration. Detection of borreliacidal antibody by membrane filtration was performed as described previously (22). Briefly, frozen supernatants were thawed, heat inactivated (56°C for 30 min), sterilized by passage through a 0.2-μm-pore-size filter (Acrodisk; Gelman Sciences, Ann Arbor, Mich.), and serially twofold- diluted (1:1 to 1:4,096) with fresh BSK medium. Then 100-μl quantities of each dilution were transferred to 1.5-ml screw-cap tubes, and 100 μl of BSK containing 10^4 *B. burgdorferi* organisms per ml was added along with 20 μl of sterile guinea pig complement (Sigma). The contents of the tubes were then gently mixed and incubated for 24 h at 35°C. Controls included filter-sterilized supernatants obtained from suspensions of nonimmune lymph node cells and *B. burgdorferi*. Other controls included supernatants from nonimmune lymph node cells and DMEM.

After incubation, 100 μl of each suspension was removed and placed into individual 1.5-ml screw-cap tubes (Sarstedt) and 100 μl of a solution of propidium iodide (1.0 mg/ ml; Molecular Probes, Eugene, Oreg.) diluted 1:50 in sterile PBS was added. The suspensions were briefly mixed before being incubated at 56°C for 30 min to permit intercalation of the propidium iodide into the spirochetes. Then 100 μl of each sample was filtered through a 0.2-μm-pore-size Nucleopore polycarbonate membrane filter (47 mm in diameter; Whatman Nucleopore, Clifton, N.J.) under negative pressure with a single-place sterility test manifold (Millipore Corp., Bedford, Mass.) attached to a vacuum pump. The membrane filters were washed with 10 ml of sterile double-distilled H_2O, removed from the vacuum apparatus, allowed to dry, and placed onto glass microscope slides. Coverslips were placed on the filters, and they were viewed with a Laborlux S fluorescence microscope (Leitz, Wetzlar, Germany) using a 50× oil immersion objective.

The number of spirochetes on each filter was determined by viewing 20 fields (22). The borreliacidal antibody titer was defined as the reciprocal of the dilution preceding the dilution at which the number of spirochetes was equal to the control. Generally, spirochetes were uniformly dispersed throughout the fields on filters of the control supernatants.

Neutralization of borreliacidal activity. Borreliacidal assays were performed with the following modifications: 20 μl of sheep anti-mouse IgG1, IgG2a, IgG2b, or IgG3 (The Binding Site, Birmingham, United Kingdom) was added to 200 μl of a 1:1,280 dilution of heat-inactivated and filter-sterilized supernatant, and the mixture was incubated for 2 h at 37°C, clarified by centrifugation (10,000 × *g* for 10 min), and filtered through a 0.2-μm filter (Acrodisk). A 100 μL sample was then used in the borreliacidal assay.

Western immunoblotting. *B. burgdorferi* isolate 50772 organisms were grown in 1 liter of BSK medium for 6 days, pelleted by centrifugation (10,000 × *g* at 15°C for 10 min), and washed three times with PBS. The washed pellet was resuspended in 1% formalin, incubated at 32°C for 30 min with periodic mixing, washed three times with PBS (12,000 × *g* at 15°C for 10 min), and resuspended in PBS. The borrelial protein content was determined by a protein assay (Bio-Rad Laboratories, Hercules, Calif.). Spirochetes were suspended 1:2 in sodium dodecyl sulfate-polyacrylamide gel electrophoresis sample buffer (Bio-Rad) and boiled for ~3 min. *B. burgdorferi* lysate protein (150 μg) was loaded onto a preparative 12% polyacrylamide gel, and the proteins were resolved by overnight electrophoresis at ~13 mA constant current with Laemmli (18) buffer system. The proteins were transferred to a nitrocellulose membrane for 1 h at 20 V, using a semidry blotting apparatus (Bio-Rad). The nitrocellulose membrane was incubated overnight at 4°C in 5% milk dissolved in Tris-buffered saline (TBS) with 0.05% Tween 20 (TBS-T; pH 7.4) to block nonspecific reactivity, washed twice each with TBS-T and double-distilled H_2O, allowed to dry, and finally cut into strips. The strips were then incubated for 1 h with samples of the supernatants (diluted in 5% milk in TBS-T), washed three times with TBS, and subsequently incubated for 1 h with a 1:1,000 dilution of an alkaline phosphatase-conjugated goat anti-murine IgG (heavy and light-chain specific; Kirkegaard & Perry Laboratories, Gaithersburg, Md.) in 5% milk in TBS-T. This step was followed by

four washes with TBS. The strips were developed by the TMB membrane peroxidase substrate system (Kirkegaard & Perry Laboratories). Reactions were stopped after 3 minutes by addition of copious volumes of chilled double-distilled H_2O.

Absorption of anti-OspC antibody. Anti-OspC antibody from lymph node supernatants was absorbed by a modification of a previously described procedure (27). TetraLink tetrameric avidin resin (Promega) was washed with PBS, and a 1-ml volume was loaded onto a column. Then 3 μg of dialyzed biotinylated rOspC in a 1-ml volume was passed over the column, and the absorbance at 280 nm was monitored to confirm binding of OspC to the column. A 1-ml sample of day 28 supernatant was then passed over the column 10 to 15 times at 4°C to remove anti-OspC antibody. Neutralization of anti-OspC antibody was confirmed by Western immunoblotting and use of an OspC enzyme-linked immunosorbent assay (ELISA).

OspC capture ELISA. Recombinant OspC was diluted to 750 ng/ml in coating buffer (0.015 M Na_2CO_3, 0.035 M $NaHCO_3$ [pH 9.6]), and 100-μl amounts were added to individual flat-bottom microtiter wells (Corning, Corning, N.Y.). The microtiter plates were incubated at 35°C for 4 h and then at 4°C overnight. After incubation, the plates were washed three times with PBS (pH 7.2) containing 0.05% Tween 20 (PBS-T), sealed, and stored at 4°C. Before being used, the plates were blocked for 30 min at 22°C with PBS-T containing 1% bovine serum albumin and washed twice with PBS-T. Subsequently, 100-μl volumes of serial twofold dilutions of supernatant in PBS-T were added to individual wells. The plates were incubated for 1 h at 22°C and than washed three times with PBS-T. Anti-mouse IgM-horseradish peroxidase conjugate (100 μl; Kirkegaard & Perry Laboratories) diluted 1:3,000 in PBS-T was added, and the plates were incubated at 22°C for 1 h. After incubation, 100 μL of *o*-phenylendiamine phosphate (0.4 mg/ml; Sigma) was added to each well, and the plates were incubated at 22°C for 30 min. The reactions were stopped by addition of 100 μl of 1 N H_2SO_4, and the absorbance at 490 nm (model EL307 instrument; Bio-Tek Instruments, Winooski, Vt.) was immediately determined. An absorbance of >0.200 unit above that of the normal supernatant control was considered positive.

IL-6 ELISA. Sterile 96-well flat-bottom plates (Corning) were coated with 50 μl of purified rat anti-murine IL-6 monoclonal antibody (MP5-20F3 clone; PharMingen) diluted in PBS to a concentration of 4 μg/ml. After incubation at 21°C for 1 h, the wells were washed three times with PBS-T. The wells were then blocked for 2 h with 200 μl of PBS containing 10% FBS (PBS-FBS) (Serum Supreme; Bio Whittaker, Inc., Walkersville, Md.). After three washes with PBS-T, the wells were inoculated in triplicate with 50 μl of supernatant and incubated for 4 h at 21°C. They were then washed five times with PBS-T and incubated for 45 min at 21°C with 50 μl of biotinylated rat anti-murine IL-6 (MP5-32C11 clone; PharMingen) diluted in PBS-FBS to 1 μg/ml. After seven washes with PBS-T, the wells were incubated (30 min at 21°C in the dark) with 75 μl of a 1:1,000 dilution of alkaline phosphatase-conjugated streptavidin (Kirkegaard & Perry Laboratories) in PBS-FBS. After nine washes with PBS-T, the wells were developed in the dark with 100 μl of TMB one-component microwell peroxidase substrate (Kirkegaard & Perry Laboratories) for 10 min. Reactions were stopped by the addition of 100 μl of TMB one-component stop solution (Kirkegarrd & Perry Laboratories), and the absorbances (450 nm) were immediately determined. Absorbance readings of supernatants were converted into picograms per milliliter through use of a standard curve obtained from an ELISA performed with an IL-6 standard (PharMingen). The IL-6 was serially diluted twofold in DMEM plus FBS from an initial concentration of 1,000 pg/ml to 7.8 pg/ml.

Statistical analysis. A *t* test (31) was used to determine significant differences in titers of borreliacidal antibody among supernatants. The alpha level was set at 0.05 before the experiments were started.

RESULTS

Production of borreliacidal antibody with different vaccine preparations containing OspC. The purpose of this study was to determine which preparation of vaccine containing OspC maximizes the production of borreliacidal antibody. Four groups of 36 mice each were vaccinated with 30 μg of rOspC or 10^6 *B. burgdorferi* cells in the presence or absence of alum. Figure 1 shows that mice vaccinated with *B. burgdorferi* in alum produced high and relatively prolonged levels of borreliacidal antibody. Antibody was detected 7 days after vaccination, peaked on days 28 to 42, and gradually declined. Considerably less borreliacidal antibody was produced in mice vaccinated with only *B. burgdorferi*. Mice vaccinated with rOspC with or without alum produced even lower levels of borreliacidal antibody than did mice vaccinated with *B. burgdorferi*. The maximum level (titer, 320) occurred on day 14 of vaccination with rOspC in alum and rapidly declined (results not shown). Vaccination with rOspC without alum produced even lower titers of borreliacidal antibody, which were not maintained. Subsequent experiments used *B. burgdorferi* in alum to study the borreliacidal antibody response. When these studies were repeated, similar results were obtained.

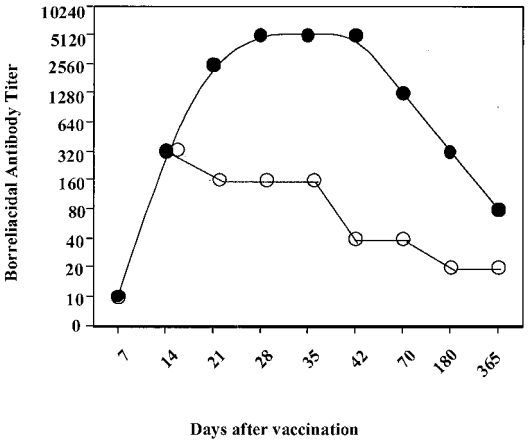

FIG. 1. Titers of borreliacidal antibody detected in sera obtained from C3H/HeJ mice vaccinated with *B. burgdorferi* isolate 50772 with (●) or without (○) alum at 7, 14, 21, 28, 35, 42, and 70 days, 6 months, and 1 year after vaccination. Borreliacidal titers were determined using *B. burgdorferi* isolate 50772, which expresses high levels of OspC but does not express OspA or OspB. In most cases (90%), the same titer was obtained when the study was repeated.

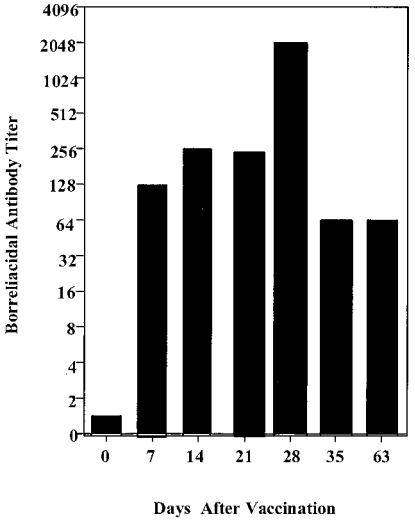

FIG. 2. Detection of borreliacidal antibody in supernatants obtained from cultures of lymph node cells containing *B. burgdorferi* isolate 50772 after incubation for 7 days. Lymph node cells were obtained from C3H/HeJ mice on days 0, 7, 14, 21, 28, 35 and 63 after vaccination with *B. burgdorferi* in alum. In most cases (90%), the same titers were obtained when the study was repeated.

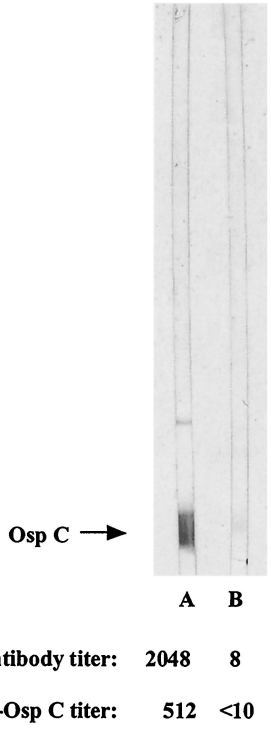

FIG. 3. Immunoblots of *B. burgdorferi* isolate 50772 after incubation with supernatant before (A) and after (B) absorption with rOspC. Supernatant was collected from lymph node cultures containing *B. burgdorferi* isolate 50772 after incubation for 7 days. The lymph node cells were obtained from C3H/HeJ mice vaccinated 28 days earlier. Borreliacidal antibody and anti-OspC titers (obtained by ELISA) are also listed before and after absorption.

Production of borreliacidal antibody in vitro by lymph node cells obtained from mice vaccinated with *B. burgdorferi* in alum. Seventy C3H/HeJ mice were vaccinated with *B. burgdorferi* in alum. Lymph node cells were collected from the mice at 0, 7, 14, 21, 28, 35, and 63 days after vaccination and cultured in vitro in the presence of 10^6 *B. burgdorferi* cells for 9 days. Supernatants were then collected on days 1, 5, 7, and 9 of in vitro cultivation, filter sterilized, and incubated with 10^4 *B. burgdorferi* cells and complement. Borreliacidal antibody (Fig. 2) was produced by lymph node cells obtained 7 days (titer, 128) after vaccination, peaked on day 28 (titer, 2048), and gradually decreased (titer, 64). In replicate experiments, supernatants obtained on day 7 of in vitro cultivation yielded maximum production of borreliacidal antibody. The peak borreliacidal antibody response also occurred with lymph node cells obtained from mice 28 days after vaccination.

Contribution of anti-OspC antibody to borreliacidal activity. Lymph nodes were harvested on day 28 after vaccination, and supernatants were obtained after 7 days of culture. The pooled supernatant was then absorbed with rOspC. Figure 3 shows that treatment of the supernatant with rOspC reduced the borreliacidal antibody titer from 2,048 to 8. Absorption of the supernatant with rOspC also decreased the rOspC ELISA titer from 510 to less than 10. Similarly, OspC antibody was not detected in the pooled supernatant after absorption with rOspC by Western immunoblotting.

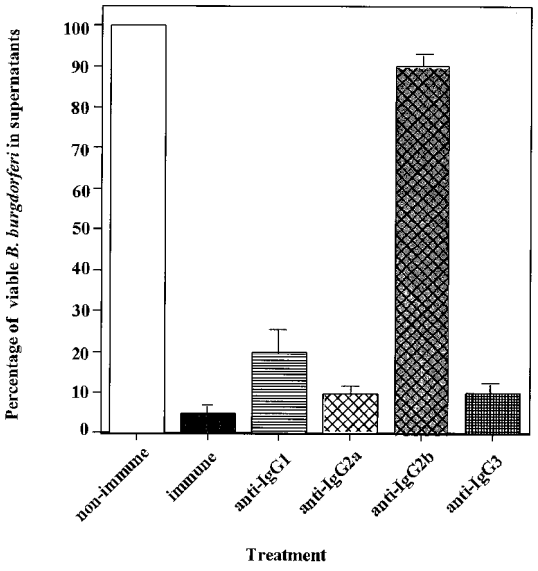

FIG. 4. Percentage of viable *B. burgdorferi* detected in supernatants obtained from immune lymph node cells, treated with anti-murine IgG1, IgG2a, IgG2b, or IgG3, compared to those obtained from nonimmune lymph node cells. Error bars represent the standard error of the mean of replicate assays.

IgG subclass of anti-OspC borreliacidal antibody. The isotype of antibody responsible for the borreliacidal response was IgG2b (Fig. 4). Anti-IgG2b antibody treatment of supernatant obtained from lymph node cells of mice vaccinated 28 days earlier abrogated the ability of the supernatant to kill *B. burgdorferi*. By contrast, treatment of supernatant with anti-IgG1, IgG2a, or IgG3 only marginally blocked the borreliacidal activity of the supernatant. No borreliacidal activity was detected with supernatant obtained from lymph node cells of nonvaccinated mice.

Modulation of the anti-OspC borreliacidal antibody response with anti-IL-6 and rIL-6. Lymph node cells from mice vaccinated 28 days earlier were cocultured with *B. burgdorferi* along with 1.0, 10, or 100 μg of anti-IL-6. Figure 5 shows that in vitro production of anti-IgG2b OspC borreliacidal antibody was inhibited by all concentrations of anti-IL-6. The borreliacidal antibody titer detected in pooled supernatant from nontreated lymph node cells was reduced from 2,048 to 64 or less with supernatant obtained from lymph node cells treated with different concentrations of anti-IL-6. Anti-OspC antibody was also not detected by Western immunoblotting with supernatant obtained from lymph node cells treated with anti-IL-6 (Fig. 6).

Figure 7 shows that production of anti-IgG2b OspC borreliacidal antibody was enhanced by treatment with rIL-6. Lymph node cells from mice vaccinated 35 days earlier cocultured with *B. burgdorferi* produced low levels of borreliacidal antibody (titer, 64). However, treatment of these lymph node cells with 0.01 or 0.1 μg of rIL-6 enhanced the activity of borreliacidal antibody 2- and 8-fold, respectively. When these experiments were replicated, similar findings were obtained.

Levels of IL-6 in cultures of lymph node cells. The levels of IL-6 were determined in supernatants from cultures of lymph node cells obtained from vaccinated mice at 0, 7, 14, 21, 28, 35,

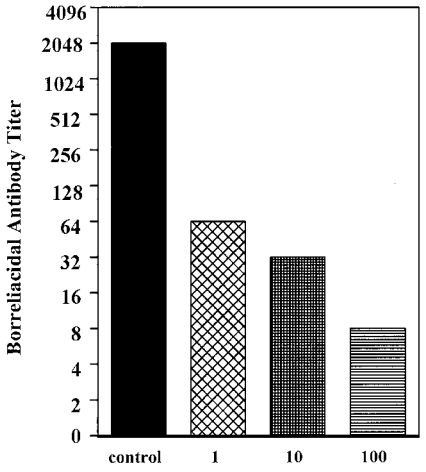

FIG. 5. Titers of borreliacidal antibody in supernatants obtained from cultures of lymph node cells containing *B. burgdorferi* isolate 50772 and 1, 10, or 100 μg of anti-IL-6 after incubation for 7 days. Lymph node cells were obtained from C3H/HeJ mice 28 days after vaccination. Control cultures were treated with a goat isotype-nonspecific IgG antibody.

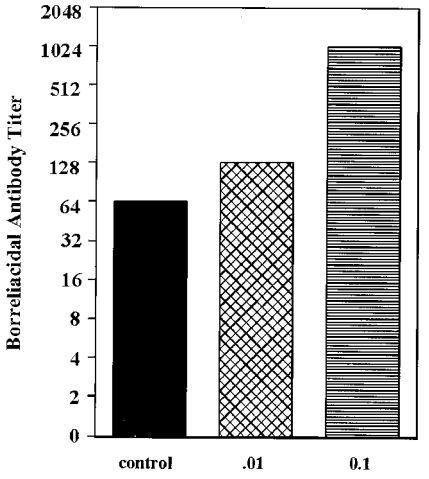

FIG. 7. Titers of borreliacidal antibody in supernatants obtained from cultures of lymph node cells containing *B. burgdorferi* isolate 50772 and 0.01 or 0.1 μg of rIL-6 after incubation for 7 days. Lymph node cells were obtained from C3H/HeJ mice 35 days after vaccination. Control cultures were treated with an equivalent volume of PBS.

and 63 days after vaccination. Table 1 shows that a low level of IL-6 was present in cultures of lymph node cells obtained on day 0. The levels of IL-6 then increased rapidly (days 7 and 14) before declining (days 21 and 28). A second increase in the levels of IL-6 was detected in supernatants of lymph node cells obtained on days 35 and 63. Only low levels of IL-6 (<25 pg/ml) were detected in lymph node cells obtained from nonvaccinated or nonvaccinated and challenged mice.

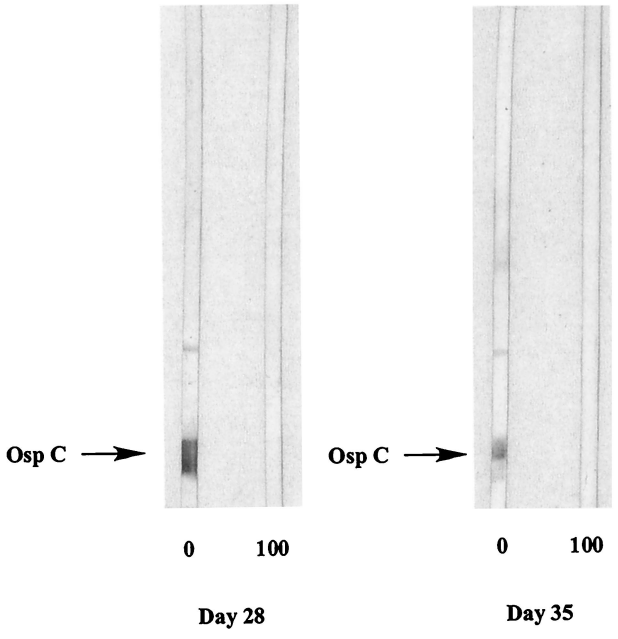

FIG. 6. Immunoblots of *B. burgdorferi* isolate 50772 after treatment with supernatant obtained from cultures of lymph node cells containing *B. burgdorferi* isolate 50772 and 100 μg of anti-murine IL-6. Supernatant was collected on day 7 of incubation. Lymph node cells were obtained from C3H/HeJ mice 28 or 35 days after vaccination. Control cultures were treated with a goat isotype-nonspecific IgG antibody.

DISCUSSION

OspC has been suggested as an alternative vaccine to OspA (14, 15, 24, 25, 27). Previous studies have shown that the levels of OspC increase on the outer surface of *B. burgdorferi* during tick attachment (28). Concomitantly, the levels of OspA and OspB are down-regulated, with little or no expression of OspA at the time of passage into the mammalian host (3, 28). Moreover, high levels of anti-OspC antibody (2, 8, 12, 13), including anti-OspC borreliacidal antibody (27), are detected in sera of patients with early Lyme borreliosis. Even after several months of infection of the host, expression of OspC is not down-regulated on *B. burgdorferi* (4). Furthermore, passive transfer of anti-OspC antibody results in resolution of established arthritis and carditis (3, 4). These findings suggest that rOspC should be considered for inclusion in a second generation of Lyme borreliosis vaccines.

There are, however, concerns about the heterogeneity (12, 32) and especially the immunogenicity (34) of OspC. Vaccination with rOspC can induce antibodies, but many of these

TABLE 1. Levels of IL-6 in supernatants obtained from cultures of lymph node cells containing *B. burgdorferi* isolate 50772 after incubation for 24 h[a]

Time (days) after vaccination	IL-6 concn (pg/ml)
0	25.0 ± 6.7
7	232.0 ± 18.5
14	137.2 ± 27.6
21	110.0 ± 12.7
28	49.5 ± 14.8
35	94.1 ± 26.6
63	157.3 ± 9.8

[a] Lymph node cells were obtained from C3H/HeJ mice on days 0, 7, 14, 21, 35, and 63 after vaccination with *B. burgdorferi* in alum.

antibodies are not involved in protection (4, 14, 34). Only a few epitopes of rOspC, perhaps just one, are responsible for the induction of antibodies that can kill *B. burgdorferi* (5, 14, 34). Production of sustained high levels of anti-OspC borreliacidal antibody is paramount for determining the potential of an OspC vaccine. Although other mechanisms of protection may be involved (24, 25, 34), we investigated only the borreliacidal antibody response induced by vaccination with OspC (5, 27).

Our results demonstrate that rOspC is a poor immunogen, even in the presence of alum, for inducing borreliacidal antibody. A low titer (<320) of anti-OspC borreliacidal antibody was produced at the peak interval of the borreliacidal antibody response after rOspC vaccination of mice. By contrast, consistently higher and sustained levels of borreliacidal antibody were produced in mice vaccinated with *B. burgdorferi*. When mice were vaccinated with *B. burgdorferi* in alum, even higher levels of borreliacidal antibody (titer, 5,120) were produced. In addition, the anti-OspC borreliacidal antibody (titer, 1,280) was maintained for 70 days. An explanation of these differences may be that native OspC on *B. burgdorferi* is processed more efficiently by antigen-processing cells than is rOspC. Recombinant OspC may have lost conformational epitopes necessary to induce high levels of borreliacidal antibody. Another explanation may be that OspC of *B. burgdorferi* 50772 is lipidated while the rOspC is not. In support, Erdile et al. (9) demonstrated that lipidation significantly increased the immunogenicity of OspA. Regardless of the mechanism of immunogenicity, rOspC needs to be improved before field trials in humans are considered.

Our results also showed that the anti-OspC borreliacidal antibody response waned rapidly. Low titers (range, 20 to 320) of borreliacidal antibody were detected in sera 2 months or less after vaccination of mice with all four preparations of OspC, despite the use of an adjuvant. Similarly, the borreliacidal antibody titer waned rapidly in supernatants obtained from cultures of immune lymph node cells. The peak anti-OspC borreliacidal antibody level (titer, 2,048) was obtained with lymph node cells obtained on day 28 after vaccination. By day 35 after vaccination, the lymph node cells produced only a low level (titer, 64) of borreliacidal antibody, despite being cultured with *B. burgdorferi* expressing high levels of OspC. Our findings suggest that immune cells become progressively less responsive to the epitope(s) of OspC responsible for the induction of borreliacidal antibody. This dampening of the protective borreliacidal antibody response may partially explain the ability of *B. burgdorferi* to persist in vivo. Vaccination with rOspC may be of limited value if this inhibitory mechanism is not neutralized or modified.

The mechanism responsible for production of anti-Osp C borreliacidal antibody does involve IL-6: When lymph node cells obtained from mice vaccinated 35 days earlier were exposed to different concentrations of IL-6, borreliacidal antibody production increased two- to eightfold. Furthermore, anti-IL-6 treatment of lymph node cells obtained 28 days after vaccination decreased the production of borreliacidal antibody by 10-fold or more. In addition, anti-OspC borreliacidal antibody was not detected in supernatants from lymph node cells obtained from mice vaccinated 28 or 35 days earlier and treated with 100 μg of anti-IL-6.

We also showed that endogenous IL-6 affects the production of anti-OspC borreliacidal antibody. Episodes of waxing and waning of IL-6 production occurred in cultures of lymph node cells obtained from vaccinated mice. The highest levels of IL-6 occurred in cultures of lymph node cells beginning to produce anti-OspC borreliacidal antibody. Once maximum production of borreliacidal antibody had occurred (day 28 after vaccination), a low level of IL-6 was detected. By day 35 after vaccination, the level of IL-6 had increased in lymph node cultures to maintain a low level of production of anti-OspC borreliacidal antibody. The number of spirochetes in the lymph node cell cultures did not influence the levels of IL-6 production. Lymph node cultures demonstrating low levels of borreliacidal antibody production contained the same number of spirochetes as did cultures of lymph node cells that caused elevated levels of killing antibody. These studies demonstrate the significance of IL-6 in the development of OspC specific humoral responses.

The decreased production of anti-OspC borreliacidal antibody by immune lymph node cells, even in the presence of high concentrations of borrelial antigen, has important implications for developing strategies to prevent Lyme borreliosis. Protracted low levels of anti-OspC borreliacidal antibody may not be adequate to eliminate *B. burgdorferi* in host tissues, especially those not readily accessible to antibody, thus promoting the chronic nature of the disease. In support, Zhong et al. (34) showed that passive transfer of antiserum to OspC resulted in resolution of infection as well as of arthritis and carditis. Since high and sustained levels of anti-OspC borreliacidal antibody are likely to constitute a critical factor in the elimination of *B. burgdorferi* from the host, the mechanism responsible for the rapid waning of borreliacidal antibody production needs to be investigated. Cytokines or other immune modulators play an important role in establishing the effectiveness and duration of protection against infection with *B. burgdorferi* (16). We found that IL-6 directly or indirectly plays an important role in the induction of anti-OspC borreliacidal antibody. Modulation of the IL-6 cytokine response or that of other cytokines that affect IL-6 may enhance the level and duration of borreliacidal antibody production.

At present we do not know which cells are responsible for the production of IL-6. IL-6 is a multifunctional cytokine secreted by a wide variety of cells including macrophages, dendritic cells, B and T lymphocytes, endothelial cells, hepatocytes, and germinal center cells (33). IL-6 receptors have been also found on B and T-lymphocytes as well as macrophages (33). Interestingly, T cells down-regulate IL-6 receptors on activation, whereas B cells acquire IL-6 receptors only at the final stages of maturation (19, 33) Ricon et al. (26) found that IL-6 was consumed during the differentiation of Th2 cells. These findings may explain our results. Thus, *B. burgdorferi* is processed by antigen-presenting cells, which release IL-6 and cause the expansion of B lymphocytes, either directly or by T-cell help, into plasma cells which produce borreliacidal antibody. Once T cells are fully activated, they may down-regulate IL-6 and the production of borreliacidal antibody. We found low levels of IL-6 in supernatants of lymph node cells with high levels of borreliacidal antibody. In contrast, high levels of IL-6 occurred only during periods (7 to 21 days after vaccination) of initial production of borreliacidal antibody and periods (35 to 63 days after vaccination) when low levels of

production of killing antibody were maintained. The mechanisms by which IL-6 is regulated need to be elucidated if the rapid waning of the borreliacidal antibody response is to be prevented.

The borreliacidal antibody response detected in vitro was due to the production of anti-OspC borreliacidal antibody. Absorption of supernatant from cultures of lymph node cells from mice vaccinated 28 days earlier with rOspC decreased the borreliacidal antibody titer from 2,048 to 8. Similarly, the rOspC ELISA titer was decreased in absorbed supernatant from 512 to less than 10. Most importantly, the anti-OspC borreliacidal antibody was due primarily to the production of IgG2b antibody. These findings suggest that OspC expresses borreliacidal epitopes that preferentially promote the IgG2b subclass. Typically, antibodies produced during a secondary immune response are of the IgG1, IgG2b and IgG3 subclasses (17). An association between IgG2b production and IL-6 has been shown (1). An advantage of IL-6-induced borreliacidal IgG2b antibody is that IgG2b fixes complement and bind Fc receptors (17, 34). Thus, OspC induces antibody with the potential to eliminate spirochetes by complement-dependent antibody-mediated lysis and by Fc-mediated phagocytosis. In contrast, Munson et al. (22) demonstrated that vaccination with OspA induces primarily IgG1, which does not bind complement. These findings suggest that OspC would be a more effective immunogen, since vaccination with OspC induces predominantly IgG2b, complement-fixing borreliacidal antibody.

In summary, we found that IL-6 plays a major role in the induction of a secondary anti-Osp C borreliacidal IgG2b antibody response. Our studies further support OspC as an alternate vaccine candidate; however, modification of the cytokine response, particularly IL-6, may be necessary to increase the duration of high levels of borreliacidal anti-OspC production. It is possible that other cytokines may interfere with IL-6 and the borreliacidal antibody response, since cytokines can act antagonistically (16). Characterization of the cytokine-induced mechanisms induced by *B. burgdorferi* or subunit vaccines will facilitate the development of a Lyme borreliosis vaccine with a prolonged duration of protection.

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