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Protective Immunity Elicited by Recombinant Bacille Calmette-Guerin (BCG) Expressing Outer Surface Protein A (OspA) Lipoprotein: A Candidate Lyme Disease Vaccine

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Summary

The current vaccine against tuberculosis, *Mycobacterium bovis* strain bacille Calmette-Guerin (BCG), offers potential advantages as a live, innately immunogenic vaccine vehicle for the expression and delivery of protective recombinant antigens (Stover, C. K., V. F. de la Cruz, T. R. Fuerst, J. E. Burlein, L. A. Benson, L. T. Bennett, G. P. Bansal, J. F. Young, M. H. Lee, G. F. Hatfull et al. 1991. *Nature [Lond].* 351:456; Jacobs, W. R., Jr., S. B. Snapper, L. Lugosi and B. R. Bloom. 1990. *Curr. Top. Microbiol. Immunol.* 155:153; Jacobs, W. R., M. Tuckman, and B. R. Bloom. 1987. *Nature [Lond.].* 327:532); but as an attenuated intracellular bacterium residing in macrophages, BCG would seem to be best suited for eliciting cellular responses and not humoral responses. Since bacterial lipoproteins are often among the most immunogenic of bacterial antigens, we tested whether BCG expression of a target antigen as a membrane-associated lipoprotein could enhance the potential for a recombinant BCG vaccine to elicit high-titered protective antibody responses to target antigens. Immunization of mice with recombinant BCG vaccines expressing the outer surface protein A (OspA) antigen of *Borrelia burgdorferi* as a membrane-associated lipoprotein resulted in protective antibody responses that were 100–1,000-fold higher than responses elicited by immunization with recombinant BCG expressing OspA cytoplasmically or as a secreted fusion protein. Furthermore, these improved antibody responses were observed in heterogeneous mouse strains that vary in their immune responsiveness to OspA and sensitivity to BCG growth. Thus, expression of protective antigens as chimeric membrane-associated lipoproteins on recombinant BCG may result in the generation of new candidate vaccines against Lyme borreliosis and other human or veterinary diseases where humoral immunity is the protective response.

Bacille Calmette-Guerin (BCG)[1] an attenuated bovine tubercle bacillus used as a vaccine for tuberculosis, offers unique advantages for development as a multivalent vaccine vehicle for other human pathogens (1–5). In initial studies recombinant BCG was shown to elicit both humoral and cellular responses to heterologous target antigens such as β-galactosidase, expressed cytoplasmically as *hsp*60-*lacZ* gene fusions (1). Subsequent studies in this laboratory have further demonstrated cytoplasmic expression of numerous antigens in rBCG, but expression levels have proven to be highly variable from antigen to antigen, and humoral responses to some cytoplasmically expressed antigens, including some antigens expressed at high levels (e.g., HIV reverse transcriptase), have been difficult to achieve. Furthermore, humoral responses to most target antigens expressed in the cytoplasm of rBCG show substantial heterogeneity among different inbred mouse strains, and even within individuals of a single strain (our unpublished data). Therefore, approaches to increase the relative immunogenicity of target antigens expressed at lower levels in rBCG were considered in an effort to enhance humoral responses elicited by rBCG. Since abundant bacterial surface proteins are often highly immunogenic, other investigators developing *Salmonella* and *Escherichia coli* vaccine vehicles have targeted small antigens or epitopes to the bacterial surface by expression as fusion proteins with flagella, fimbriae, or major outer membrane protein A (OmpA) and λ phage receptor (LamB) (6–10). As comparable immunogenic pro-

[1] *Abbreviations used in this paper:* BCG, bacille Calmette-Guerin; OspA, outer surface protein A; RBS, ribosomal binding site; T, Tween; TX, Triton X.

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tein surface structures have not yet been characterized in mycobacteria, we have chosen to use signal peptides derived from lipoproteins (11) to direct export and membrane-associated surface expression of target antigens in BCG. In addition to the potential for improved antigen presentation by surface expression on rBCG, lipid acylation can dramatically increase the ability of synthetic peptides (12, 13) to elicit immune responses. Moreover, lipoproteins of bacterial pathogens are highly immunogenic in vivo and some bacterial lipoproteins have been implicated as protective antigens (14–22).

Possibly the most notable of promising protective bacterial lipoprotein antigens is the outer surface protein A (OspA) antigen of *Borrelia burgdorferi*, the causative agent of Lyme disease (23–25). A number of studies have implicated OspA as a protective antigen and OspA-specific antibodies as protective against Lyme borreliosis in the mouse model (26–29). Passive transfer of monoclonal and polyclonal sera specific for OspA was shown to protect mice against challenge with *B. burgdorferi* (26, 27). Removal of antibodies specific for OspA greatly diminished the protective component in polyclonal sera generated by immunization with whole killed *Borrelia* (28). Studies using non-lipid acylated OspA-glutathione *S*-transferase (OspA-GST) fusion protein in active immunizations have also demonstrated promising protection in the mouse model for Lyme borreliosis; however, protection required repeated immunizations with large doses of OspA-GST and formulation with CFA, which is unsuitable for human use (27–29). As a lipoprotein, OspA is insoluble and not expressed well in *E. coli*; accordingly, OspA can be unwieldy for immunogenicity studies in the laboratory or large-scale production as a subunit vaccine (30). Recently, however, it was demonstrated that purified OspA lipoprotein (L-OspA) is profoundly more immunogenic than non-lipid acylated OspA (NL-OspA), even in the absence of adjuvant (31). Since OspA is a bacterial lipoprotein amenable to membrane translocation, we used the *osp*A gene to test the function and utility of the rBCG lipoprotein expression vectors (LPE vectors) using mycobacterial lipoprotein signal peptides that can enable the expression and export of heterologous chimeric lipoproteins to the membrane of rBCG.

In this study we report a comparison of immune responses elicited by immunization with rBCG expressing the OspA antigen cytoplasmically, as a secreted fusion protein or as a membrane-associated lipoprotein. Immunization with rBCG expressing OspA as a chimeric lipoprotein, but not as a cytoplasmic or secreted protein, resulted in high-titered antibody responses to OspA in all mouse strains tested, including outbred mice. These OspA-specific antibodies inhibited growth of *B. burgdorferi* in vitro and in vivo, and were protective in a mouse model for Lyme borreliosis. This study is a first test for a strategy to deliver protective membrane-associated lipoproteins on live recombinant BCG vaccines, and has resulted in the development of a candidate vaccine against Lyme disease.

Materials and Methods

Construction of BCG/E. coli Shuttle Expression Vectors for Expression of OspA. All expression vectors described in this study are derivatives of plasmid pMV206 described by Stover et al. (1) and include DNA cassettes encoding kanamycin resistance, an *E. coli* origin of replication, a mycobacterial plasmid replicon derived from *Mycobacterium fortuitum* plasmid pAL5000, and a synthetic multiple cloning site (MCS). Plasmid pMV251 is a derivative of pMV261 (1) with overlapping NcoI and BamHI restriction sites at the hsp60 start codon. A DNA segment spanning the *M. tuberculosis* (strain H37Rv) 19-kD antigen gene promoter, ribosomal binding site (RBS), secretion signal, and the first six codons of the mature processed protein was amplified by PCR and cloned between the XbaI and BamHI sites of pMV206 to construct plasmid p19PS. A DNA segment including RBS and the structural gene of the BCG α antigen was amplified by PCR without the α antigen stop codon and cloned between the XbaI and BamHI sites in the MCS of plasmid pRB26 (a derivative of pMV206 containing the BCG hsp60 promoter) to yield plasmid pAB26. An *ospA* gene segment encoding the *ospA* gene without the 5′ region encoding the signal peptide (*ospA*Δ1–57) was amplified by PCR using oligonucleotides based on published sequence data for *B. burgdorferi* strain B31 (35210; American Type Culture Collection (Rockville, MD) OspA (25). These primers included a unique BamHI (5′ *osp*A primer) or SalI site (3′ *ospA* primer) at their 5′ ends to facilitate directional cloning. The resulting OspA PCR gene segment was digested with BamHI and SalI to generate cohesive ends at the 5′ and 3′ ends, respectively, and cloned in-frame between the BamHI and SalI sites of plasmids pMV261, pAB26, and p19PS to yield plasmids pMV261::*osp*A, pAB26::*osp*A, and p19PS::*osp*A. A complete *osp*A gene (including the signal region) was similarly constructed with 5′ NcoI site at the start codon and cloned between the NcoI and SalI sites of plasmid pMV251 to yield pMV251::*osp*A.

Culture and Transformation of BCG. All liquid culture of BCG substrain Pasteur 1173 A2 was accomplished at 37°C in stationary tissue culture flasks (25 cm^2 with 5–10 ml or 75 cm^2 with 15–25 ml) or roller bottles (490 cm^2 with 100–200 ml or 850 cm^2 with 200–500 ml) with Dubos media (Difco Laboratories, Detroit, MI) supplemented with a 10% volume of albumin dextrose (AD) enrichment consisting of 5% BSA fraction V (Sigma Chemical Co., St. Louis, MO), 2% dextrose, and 0.85% sodium chloride. Liquid culture media included 0.02% Tween 80 (T80) to prevent clumping of BCG cells. BCG colonies were grown at 37°C on Middlebrook 7H10 agar media (Difco Laboratories) supplemented with 10% volume of AD enrichment. For transformation, BCG cultures were grown to densities of $\sim 10^7$ CFU/ml, sedimented at 4,000 *g*, and washed twice by resuspension and centrifugation (4,000 *g*) in 10% glycerol at 4°C, and finally resuspended in 5% of the original culture volume of cold 10% glycerol. 200 μl of the cold BCG suspension was mixed with plasmid DNA (50–500 ng) in a prechilled 0.2-cm electroporation cuvette and transformed using a gene pulser electroporator at 2.5 kV, 25 μf, and 1,000 Ω (Bio-Rad Laboratories, Richmond, CA). After electroporation, 50 μl of 5× Dubos media was added to the BCG-DNA suspension and this mixture was incubated at 37°C for 1 h before plating on Middlebrook 7H10 plates supplemented with AD enrichment and kanamycin (15 μg/ml).

Analysis of OspA Expression in rBCG. BCG transformants were grown to mid-logarithmic phase in Dubos liquid media containing kanamycin (15 μg/ml), sedimented at 4,000 *g*, and prepared for Western blot analysis by washing in a PBS + 0.05% T80 20-fold concentration by centrifugation and sonication in radioimmunoprecipitation assay (RIPA) buffer (1% NP-40, 0.5% deoxycholate, 0.1% SDS, 50 mM Tris (pH 8.0). Culture lysates approximately equivalent to 5×10^6 bacteria were analyzed by SDS-PAGE and Western blot with the OspA-specific mAb H5332 (18). Expres-

sion of OspA by rBCG was compared to a *B. burgdorferi* lysate derived from strain B31 and to purified OspA lipoprotein, kindly provided by Dr. L. Erdile (Connaught Laboratories Inc., Swiftwater, PA). Protein bands reacting with H5332 were visualized after incubation with a secondary antibody (goat anti-mouse IgG conjugated to horseradish peroxidase) using the enhanced chemiluminescent detection (ECL) system (Amersham Corp., Arlington Heights, IL) according to manufacturers specifications.

Localization of OspA Fusion Proteins in Recombinant BCG by Triton X-114 Phase Partitioning. rBCG cells were sedimented from cultures, suspended in PBS, and cell suspensions were adjusted to equivalent densities. Cells were disrupted by sonication and membranes were solubilized at 4°C by the addition of Triton X-114 (TX-114) to 2% (vol/vol). Insoluble material (cell wall-enriched fraction) was sedimented by centrifugation at 100,000 *g* and the supernatant was subjected to detergent phase partitioning (32). After briefly warming (37°C) the TX-114 solutions, separation of aqueous and detergent phases was achieved by a short centrifugation. These two phases were back-extracted three times (33), and proteins in representative samples were precipitated by the addition of nine volumes of acetone. A portion of each culture supernatant was concentrated by ultrafiltration (Centricon 30; Amicon Corp., Danvers, MA). Samples representing fivefold concentrated culture volume equivalents were subjected to SDS-PAGE, transferred to nitrocellulose, and blotted with anti-OspA mAb H5332. Endogenous Hsp60, BCG α antigen (αAg), and Mtb19 BCG homologous antigens were detected by Western blot with mAbs IT13, HYT27, and HYT6, respectively (kindly supplied by T. Shinnick, Centers for Disease Control, Atlanta, GA; and J. Ivanyi, Medical Research Council, Hammersmith Hospital, London, UK), as part of the United Nations Development Program/World Bank/World Health Organization Special Programme for Research and Training in Tropical diseases.

Surface Labeling and Flow Cytometry of rBCG Expressing OspA. Approximately 2×10^9 rBCG grown in Dubos-AD were harvested by centrifugation. The pelleted rBCG were washed with 10 ml of PBS-T80 (pH 7.4), resuspended in 5 ml PBS-T80, then fixed with an equal volume of 4% paraformaldehyde in PBS-T80 for 10 min at 4°C. Fixed rBCG were pelleted and washed twice with 5 ml PBS-T80, then resuspended in a 1-ml volume of PBS-T80 for treatment with α-OspA polyclonal sera. The anti-OspA polyclonal serum was generated by hyperimmunization of rabbits with purified OspA lipoprotein in IFA. Possible background reactivity was removed by exhaustive preadsorption with BCG lysate immobilized on nitrocellulose membranes. This preadsorbed anti-OspA polyclonal sera was added to the fixed rBCG cell suspension at a final dilution of 1:200 and incubated for 30 min at room temperature and 30 min on ice. The rBCG were pelleted by centrifugation, washed twice with 0.5 ml PBS-T80, and resuspended in 1 ml PBS-T80. Goat anti-rabbit FITC-conjugated secondary antibody (GIBCO BRL, Gaithersburg, MD) was added to a final dilution of 1:50 and incubated for 30 min on ice. The rBCG–antibody complex was pelleted by centrifugation, washed twice with 1 ml PBS-T80, and resuspended in 2 ml PBS-T80. Labeled rBCG were mildly sonicated to disperse clumped cells and dilutions were analyzed by flow cytometry on a FACScan® (Becton Dickinson & Co., Mountain View, CA).

rBCG Preparation and Immunization. rBCG cultures were grown to ODs of ~1.2 A_{600} U (~2×10^8 CFU/ml), concentrated 20-fold by sedimentation at 4,000 *g*, and resuspended in 15% glycerol. 1-ml vials of the rBCG glycerol suspension were gradually frozen at a rate of −1°C/min from 4 to −40°C. The frozen rBCG were stored at −70°C until use. CFU were assayed by plating mildly sonicated dilutions of a thawed material on Middlebrook 7H10 plates + kanamycin as described above.

Female 4–6-wk-old BALB/c and C3H/HeJ inbred mice (The Jackson Laboratory, Bar Harbor, ME) and outbred Swiss Webster mice (Charles River Labs., Wilmington, MA) were immunized (priming and boosting) by the intraperitoneal route with 10^6 rBCG CFU in 100 μl of PBS-T80.

Immunogenicity of rBCG-ospA Constructs. Sera were collected from the tail veins of immunized mice at 4-wk intervals and pooled for each group to monitor antibody responses by ELISAs with *B. burgdorferi* (strain B31) spirochetes. ELISA plates (Immulon 1) were coated with 50 μl of whole *Borrelia* resuspended in 0.1 M carbonate buffer (pH 9.6) at 10 μg/ml protein or with 100 μl of recombinant 0.1 μg/ml OspA in the same carbonate buffer and incubated for 2–4 h at room temperature or overnight at 4°C. The antigen solution was removed and plates were incubated with blocking solution (0.5% BSA and 0.5% nonfat dry milk) in PBS with 0.1% Tween-20 (PBS-T20) for 1 h at room temperature. Twofold serial dilutions of serum starting at 1:200 were made in blocking solution and 50 μl of each dilution was added to duplicate wells of the antigen-coated plate. After an incubation at room temperature for 1 h, the coated plates were washed with PBS-T20 and incubated with 50 μl of a 1:1,000 PBS-T20 dilution of peroxidase-conjugated goat anti-mouse IgG (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD) secondary antibody for 1 h. Color was developed with 2,2′-azino-di[3-ethyl-benzthiazoline sulfonate (6)] substrate reagent (Kirkegaard & Perry Laboratories, Inc.) and measured by absorbance at 405 nm on an ELISA reader (Dynatech). Endpoint titers were defined as the highest dilution at which the A_{405} values were twice the values for preimmune sera diluted 1:200 in blocking solution.

Western immunoblot analysis was performed with lysates of whole *B. burgdorferi*. After separation by SDS-PAGE and transfer to nitrocellulose, individual lanes were incubated for 1 h with pooled serum diluted 1:500 in PBS-T20, washed with PBS-T20, reacted with horseradish peroxidase–conjugated goat anti-mouse secondary antibody for 30 min, and developed with the ECL chemiluminescent substrate according to manufacturer's instructions (Amersham Corp.).

In Vitro Growth Inhibition Assay. In vitro growth inhibition antibody titers against the B31 strain of *B. burgdorferi* were determined as described elsewhere (34) for pooled sera from rBCG-immunized mice in two independent experiments. Wells of a 96-well, flat-bottomed microtiter plate were seeded with 2×10^6 spirochetes in 0.1 ml of BSK-II media derived from a late log-phase culture of a high-passage-number isolate of *B. burgdorferi* B31 (35210; American Type Culture Collection) grown in the same medium. Before addition to wells mouse serum was heat inactivated (growth inhibition in this assay is complement independent) and filtered through a 0.22-μm filter to sterilize the serum. Twofold serial serum dilutions starting at 1:8 were added to duplicate culture wells in the 96-well plates, and the plates were sealed and incubated at 34°C for 72 h in a 1% CO_2 atmosphere. Bacterial growth in each well was monitored by color changes in the phenol red indicator dye with a 580 ELISA reader at 490 nm (Dynatech Labs. Inc., Chantilly, VA). Spirochete growth was also confirmed by phase contrast microscopy in control wells. As a control for specificity of serum growth inhibition *B. hermsii* strain HSI (35209; American Type Culture Collection) was used in the same experiments.

B. burgdorferi Challenge of Mice Immunized with rBCG-OspA Vaccines. Challenge doses were derived by expansion of a single colony of the low-passage *B. burgdorferi* Sh.2 strain (35) and were administered 5 wk after the rBCG booster immunization. Immunized

and control mice were challenged intraperitoneally with 10^6 spirochetes in experiment 1. In experiment 2, mice were inoculated intradermally at the base of the tail with 10^4 spirochetes. This represented ~100 ID_{50} units of the *B. burgdorferi* Sh.2 strain. Mice were killed 14 d after challenge, and plasma and bladder, heart, and joint tissues were harvested, minced, and cultured in BSK-II media as described previously (31). Cultures were monitored through day 14 by phase contrast microscopy for the presence of spirochetes. The presence of one or more spirochetes per 20 high-power fields in any one of the blood or tissue cultures was scored as a positive infection.

Results

Expression of Membrane-associated OspA Lipoprotein on Recombinant BCG. Initial studies in this laboratory with an rBCG vaccine expressing OspA at high levels in the cytoplasm of rBCG resulted in modest anti-OspA humoral responses (36). We therefore initiated strategies to express OspA as an exported membrane-associated lipoprotein with the aim of enhancing OspA immunogenicity in rBCG. Mice and humans immunized with BCG or infected with *Mycobacterium tuberculosis* often exhibit higher titered antibody responses to a 19-kD antigen (Mtb19) than to the numerous other antigens of these mycobacteria (37, 38). DNA sequence analysis of the gene encoding the immunogenic Mtb19 antigen of *M. tuberculosis* indicated that the hydrophobic NH_2-terminal region was a lipoprotein signal peptide (39). Subsequent biochemical analysis confirmed that the Mtb19 protein was lipid acylated by *M. tuberculosis*, presumably during the export process (40). Accordingly, we constructed expression vectors using the Mtb19 lipoprotein signal peptide in an attempt to direct the expression, export, and lipid acylation of heterologous antigens, such as OspA, by recombinant BCG. For comparison, an expression vector was also constructed for expression of fusion proteins with the αAg, which is a 32-kD secreted protein found in the culture filtrate BCG85 complex of BCG and other mycobacteria (41–45). The αAg homologue of *M. kansasii* has been used previously as a carrier protein to secrete an HIV-1 Gag peptide from rBCG, but immune responses using this strategy have not been reported (44, 45).

As a bacterial lipoprotein with promising vaccine efficacy, OspA was chosen as an appropriate antigen to test the utility of the rBCG lipoprotein expression vectors (LPE vectors). With the aim of comparing immune responses to rBCG expressing OspA as a cytoplasmic protein (C-OspA), a secreted protein (S-OspA), or as a membrane-associated lipoprotein (L-OspA), we cloned an *osp*A gene segment excluding the 5′ signal peptide region (OspA Δ1–18) into rBCG expression vectors pMV261, pAB26, and p19PS, respectively (Fig. 1). A complete *osp*A gene segment including its native signal peptide was also cloned into rBCG expression vector pMV251 in an attempt to derive expression of a complete and authentic OspA lipoprotein in rBCG (Fig. 1). Recombinant gene products that were reactive with a mAb specific for OspA were obtained at the predicted molecular weights for all *osp*A constructs described above (Fig. 2). Expression levels of the OspA protein from the rBCG-pMV261::*osp*A construct were estimated to be in excess of 10% total rBCG protein (~20 ng/10^6 rBCG), while expression levels for OspA fusions to the Mtb19 signal peptide, the αAg, or the native OspA signal peptide were substantially lower (~1–5 ng/10^6 rBCG). Surprisingly, chimeric OspA lipoproteins and the αAg-OspA fusion protein were seemingly more reactive in Western blot analyses with the OspA-specific H5332 mAb even though these gene products were expressed at much lower levels than the fusion protein expressed by pMV261::*osp*A (Fig. 2). This observation is probably explained by increased avidity of the αAg-OspA fusion protein and lipid acylated OspA lipoprotein for the nitrocellulose membrane, as described in a previous study (30), since nonlipid acylated OspA has been shown to be antigenically equivalent to OspA lipoprotein (31).

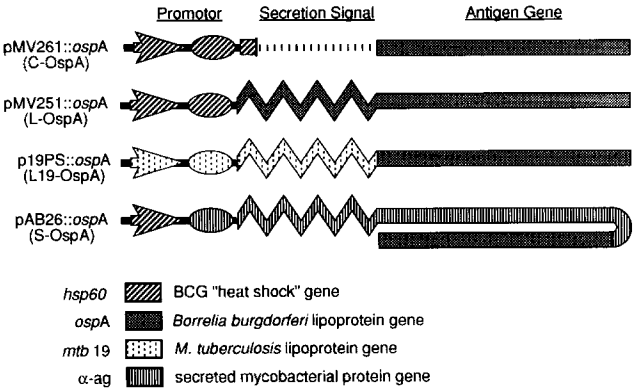

Figure 1. BCG/*E. coli* shuttle expression vectors used to express *osp*A.

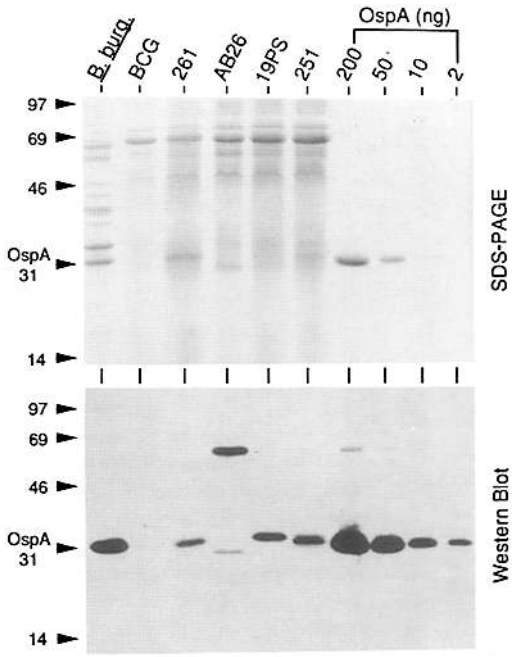

Figure 2. Expression of *osp*A in rBCG. Plasmids constructed in *E. coli* as described in Fig. 1 were transformed into BCG substrain Pasteur 1173A2 by electroporation. Transformants were grown and ~5 × 10^6 rBCG bacteria were harvested and analyzed by SDS-PAGE (*top*) and Western blot with OspA-specific mAb H5332 (*bottom*) for comparison to a *B. burgdorferi* lysate, and purified OspA lipoprotein.

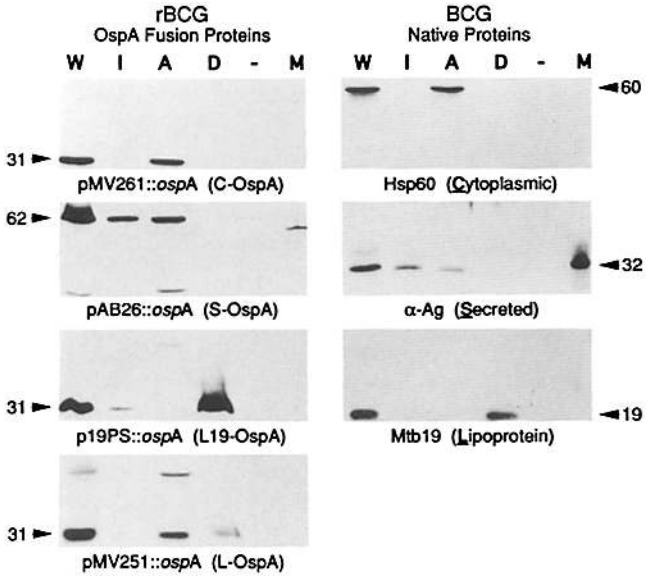

Figure 3. Localization of OspA fusion proteins in recombinant BCG by TX-114 phase partitioning. rBCG lysates were fractionated by centrifugation and TX-114 phase partitioning as described in Methods. Samples representing fivefold concentrated culture volume equivalents were subjected to SDS-PAGE, electroblotted to nitrocellulose, and reacted with anti-OspA mAb H5332 (*left*). Identically processed fractions from nonrecombinant BCG were blotted with appropriate mAbs specific for the BCG or *M. tuberculosis* hsp60 (IT13), α-Ag (HYT27), or Mtb19 antigens (HYT6) to determine the cellular location of the endogenous fusion partners. Lane *W*, whole cell sonicate; lane *I*, TX-114-insoluble material (cell wall enriched); lane *A*, aqueous phase (cytosol enriched); lane *D*, detergent phase (membrane/lipoprotein enriched); lane *M*, fivefold concentrated culture medium (secreted). Positions of chimeric OspA protein bands are indicated by arrows at 31 kD. Positions of the endogenous BCG proteins used as fusion partners are indicated on the right with the molecular masses (in kD).

To determine if expression of *osp*A genes from the lipoprotein expression vectors actually resulted in export and lipid acylation of recombinant OspA, exponentially growing cultures of the rBCG-OspA recombinants and nonrecombinant BCG were subjected to cell fractionation and TX-114 detergent phase partitioning analysis to enrich for membrane-associated lipoproteins (32, 33). The OspA gene product encoded by pMV261::*osp*A (C-OspA) was found exclusively in the aqueous cytosolic fraction and correlated with the restricted cytoplasmic location of the BCG Hsp60 (Fig. 3). The αAg-*osp*A gene product (S-OspA) expressed by plasmid pAB26::*osp*A as well as the endogenous BCG αAg were found in the insoluble cell wall–enriched fraction, aqueous cytosolic fraction, and the extracellular media fraction, but not in the detergent-soluble membrane-enriched fraction. The presence of the αAg-OspA fusion protein or αAg in the rBCG culture media was not due to rBCG autolysis inasmuch as hsp60, which we determined to be exclusively cytosolic in exponentially growing cultures, was not found in the culture media. Compared with the native BCG αAg, a smaller fraction of the total S-OspA fusion protein pool was secreted into the media, while a larger portion was found in the cell wall–enriched insoluble fraction. This suggested that some of the exported αAg fusion protein associated with the cell wall, although the possibility that some of the αAg-OspA is expressed as an insoluble cytoplasmic inclusion can not be excluded by these data. Substitution of the Mtb19 signal peptide for the OspA signal peptide resulted in expression of a chimeric OspA (L19-OspA) that was almost exclusively located in the detergent-soluble fraction, which is highly enriched for lipophilic proteins. This finding correlated with the location of the endogenous membrane-associated Mtb19 lipoprotein BCG homologue and indicated that fusion of the Mtb19 signal peptide to OspA did indeed direct efficient export and posttranslational modification of the chimeric L19-OspA protein to the membrane of rBCG. This result was in contrast to the predominantly cytosolic location for the gene product encoded by pMV251::*osp*A (L-OspA), where a very minimal amount of OspA was found in the detergent-soluble lipophilic fraction. This result was possibly indicative of inefficient or incomplete processing of the foreign *Borrelia* signal peptide by the mycobacterial lipoprotein–specific signal peptidase II.

The rBCG-OspA bacilli were also analyzed by flow cytometry to confirm predictions of the cellular localization of the recombinant OspA gene products (Fig. 4). rBCG expressing L19-OspA, L-OspA, and S-OspA, from plasmids p19PS::*osp*A, pMV251::*osp*A, and pAB26::*osp*A, respectively, all demonstrated increased surface fluorescence with anti-OspA sera when compared with nonrecombinant BCG or rBCG expressing C-OspA from plasmid pMV261::*osp*A. Although discernibly less than that of rBCG expressing L19-OspA, the surface fluorescence exhibited by rBCG with plasmid pMV251::*osp*A was somewhat surprising in light of the finding that the majority of this OspA protein was cytosolic. Nevertheless, this finding was in general agreement with the TX-114 fractionation analysis, which indicated that a small amount of lipophilic L-OspA was associated with the BCG membrane as a lipophilic protein. The rBCG expressing the S-OspA from vector pAB26::*osp*A also exhibited surface fluorescence, thus confirming the finding that the αAg-OspA fusion protein found in the TX-114-insoluble fraction was cell wall associated and not derived primarily from insoluble cytoplasmic inclusion bodies. Further confirmation of OspA surface localization by fusion to the Mtb19 lipoprotein signal peptide or αAg in rBCG was obtained by immunoelectron microscopy of rBCG thin sections (data not shown, S. Hanson et al., manuscript in preparation). These data, taken together with the fractionation and TX-114 phase partitioning analysis, confirm that it is possible to export OspA to the exterior of rBCG as a chimeric membrane-associated lipoprotein by fusion to the Mtb19 lipoprotein signal or as a secreted and cell wall–associated protein by fusion to the αAg.

Enhanced Humoral Response to rBCG Membrane-associated OspA. A survey of 24 inbred and outbred mouse strains was performed to determine relative humoral responsiveness to recombinant OspA expressed in the cytoplasm of rBCG and to determine whether the mouse BCG allele, which confers resistance or sensitivity to in vivo growth of BCG and other intracellular pathogens, had any impact on responsiveness to rBCG-expressed OspA (46). OspA-specific antibody responses

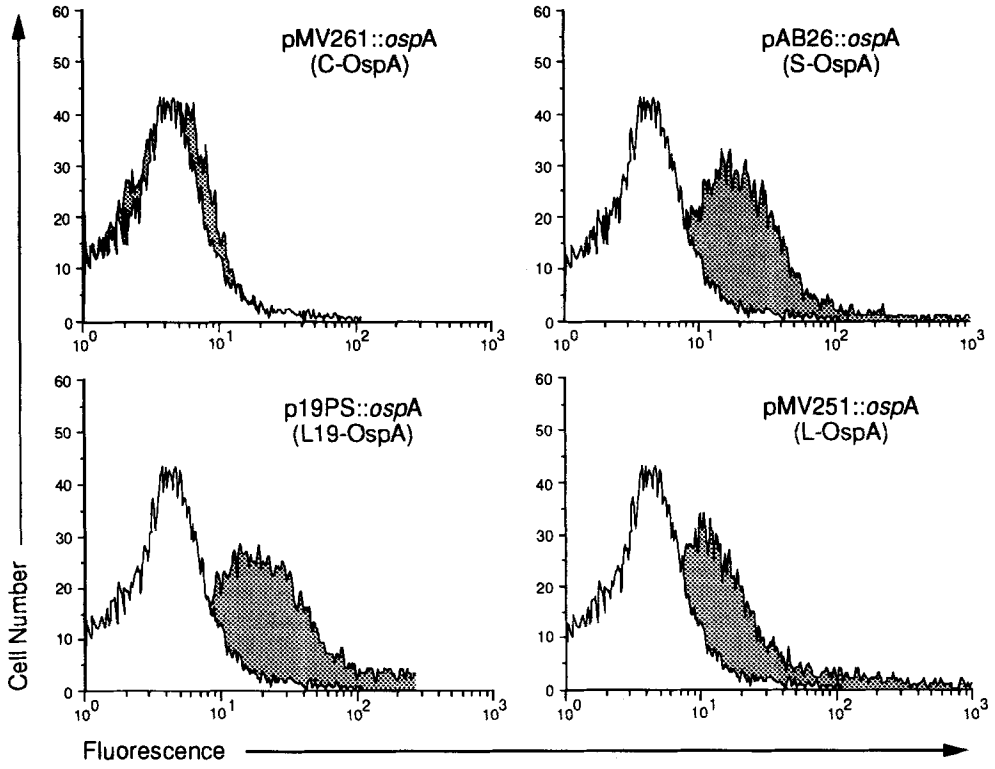

Figure 4. Surface labeling and flow cytometry of rBCG expressing OspA. rBCG containing the designated plasmids and expressing the designated chimeric OspA gene products (*shaded histograms*) are compared to nonrecombinant BCG (*open histograms*).

resulting from immunization with rBCG pMV261::*ospA* expressing C-OspA did not correlate with the mouse BCG allele, as responding mouse strains carrying either BCG-s (sensitive) and BCG-r (resistant) alleles were observed (46). C3H/He, BALB/c, and outbred Swiss Webster mice responded to rBCG-pMV261::*ospA* immunization with high (not requiring boosting), intermediate (requiring boosting), and low (barely detectable after boosting) anti-OspA responses, respectively. In addition to their variable responses to OspA, these mouse strains differ with respect to their resistance to BCG growth; BALB/c mice carry the BCG-s (sensitive) allele and both C3H and outbred Swiss Webster mice are BCG-r (resistant). Thus, these three mouse strains provided a manageable representative sampling of mouse phenotypes for comparison of rBCG vaccines expressing OspA as a cytoplasmic protein, a membrane-associated lipoprotein, or as a cell wall–associated and secreted protein. C3H/HeJ, BALB/c, and the outbred Swiss Webster mice were immunized intraperitoneally with 10^6 CFU of rBCG-OspA and pooled sera were analyzed by ELISA to whole *Borrelia* at 4, 8, 12, and 16 wk after a single immunization. All mice were boosted at 17 wk with the same rBCG vaccine used in the initial immunization and sera were analyzed for reactivity to OspA 2 wk postboost. All three mouse strains immunized with rBCG vaccines expressing OspA as lipoproteins (L-OspA and L19-OspA) exhibited strong OspA-specific antibody responses within 4–8 wk after a single rBCG-OspA immunization, as measured by ELISA to whole *Borrelia* (Fig. 5). Similar responses to rBCG

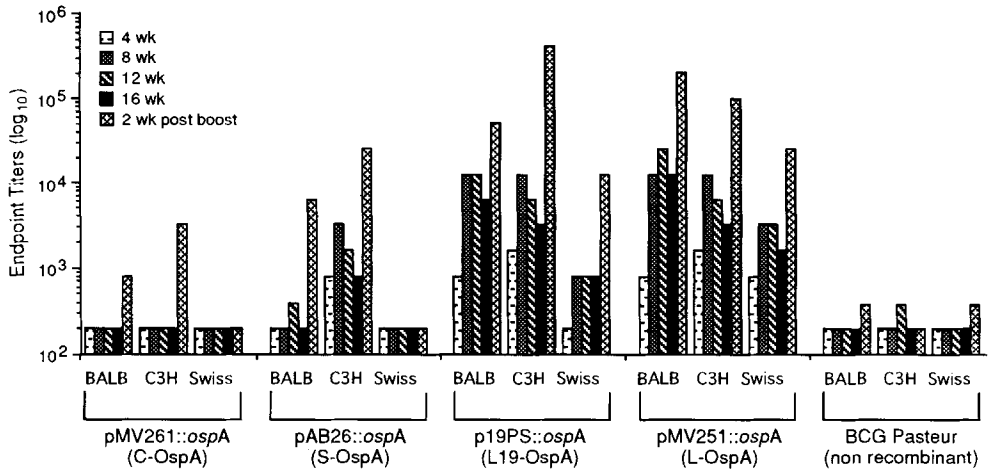

Figure 5. Comparison of immunogenicity of rBCG-*ospA* constructs (cytoplasmic, secreted, and lipid acylated). Mouse strains were immunized by the intraperitoneal route with 10^6 CFU of rBCG and boosted at 17 wk with the identical dose. Sera were collected at the indicated times, pooled, and analyzed by ELISA with whole *B. burgdorferi* (strain B31).

Table 1. *Anti-OspA Endpoint Titers (Exp. 1)*

rBCG vector* immunogen	Mean anti-OspA endpoint titer‡ mouse serum (2 wk postboost)		
	BALB	C3H	Swiss
pMV261::*ospA* (C-OspA)	400 ± 0	400 ± 0	400 ± 0
pMV251::*ospA* (L-OspA)	289,631 ± 58,341	243,550 ± 42,415	204,800 ± 0
p19PS::*ospA* (L19-OspA)	243,550 ± 42,415	819,200 ± 0	43,054 ± 7,498
pAB26::*ospA* (S-OspA)	2,691 ± 469	12,800 ± 0	951 ± 166
BCG control§	400 ± 0	200 ± 0	400 ± 0

* Vector used to express OspA gene in rBCG vaccine.
‡ ELISA to purified OspA lipoprotein.
§ Nonrecombinant BCG (Pasteur substrain).

C-OspA were not seen until after boosting. Particularly striking were the anti-OspA responses elicited by a single dose of either of the rBCG-L-OspA vaccines in the low responder outbred Swiss Webster mice. This mouse strain immunized with rBCG expressing OspA as a cytoplasmic or secreted protein did not mount anti-OspA responses even after boosting. Peak anti-OspA antibody titers >1:10^5 in BALB/c and C3H mice and 1:10^4 in Swiss mice were elicited by boosting with rBCG-L-OspA constructs. These responses were 100–1,000-fold higher than the responses induced with rBCG constructs expressing as a cytoplasmic protein or secreted surface-associated OspA-αAg fusion protein. The specificity of antibodies (pooled sera) to OspA generated by immunization with rBCG-OspA vaccines was confirmed by ELISA with purified OspA lipoprotein (Table 1) and Western blot analysis with *B. burgdorferi* lysates (Fig. 6). Analysis of individual mouse anti-OspA titers revealed that all mice immunized with rBCG-L-OspA vaccines responded comparably, while significant individual mouse–mouse variation was observed in animals immunized with rBCG vaccines expressing C-OspA or S-OspA (data not shown).

Protection against B. burgdorferi Infection with an rBCG-based Vaccine. The OspA antigen of *B. burgdorferi* has been shown to be protective in both immunocompetent and immunode-

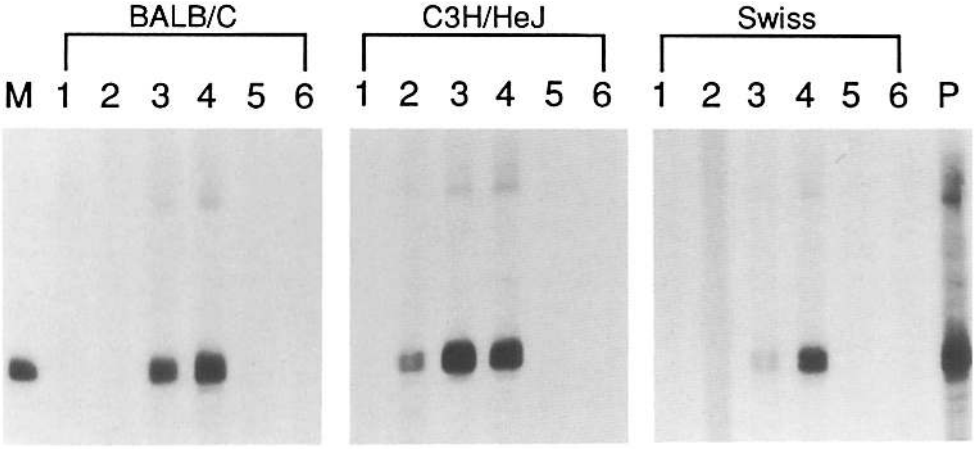

Figure 6. Western blot reactivity of antibodies elicited by rBCG-OspA vaccines. A *B. burgdorferi* lysate (strain B31) was reacted with pooled immune sera collected 2 wk after a single booster immunization from rBCG-immunized BALB/C, C3H/HeJ, or Swiss Webster mice. Pooled immune sera was derived from mice immunized with rBCG expressing OspA from vectors pMV261::*ospA* (C-OspA, lanes *1*), pAB26::*ospA* (S-OspA, lanes *2*), p19PS::*ospA* (L19-OspA, lanes *3*), pMV251::*ospA* (L-OspA, lanes *4*), from mice immunized with control nonrecombinant rBCG (BCG Pasteur, lanes *5*), or from mice bled before immunization (preimmune sera, lane *6*). Identical *B. burgdorferi* lysates were also reacted with OspA-specific mAb H5332 (lane *M*) and anti-OspA hyperimmune rabbit sera (lane *P*).

Table 2. *In Vitro Growth Inhibition Titers and In Vivo Protection against B. burgdorferi Challenge (Exp. 1)*

rBCG vector§ immunogen	In vitro growth inhibition titer‡ of mouse serum		In vivo protection: no. mice with culturable borrelia in tissues or blood/total no. mice*			
			Bladder culture		Heart culture	
	BALB	C3H	BALB	C3H	BALB	C3H
pMV261::*ospA* (C-OspA)	<8	32	5/5	3/4	1/5	0/5
pMV251::*ospA* (L-OspA)	4,096	1,024	0/5	0/5	0/5	0/5
p19PS::*ospA* (L19-OspA)	1,024	2,048	0/5	3/5	0/5	0/5
pAB261::*ospA* (S-OspA)	ND	256	4/5	3/5	1/5	1/5
BCG control‖	<8	<8	4/4	5/5	3/4	3/5

B. burgdorferi challenge used 10^6 CFU of low-passage SH.2 strain (31, 35).
* Number of mice in each group testing positive for borrelia infection (low-passage Sh.2 strain) by culture of tissues 14 d after challenge as a fraction of the total number of mice per group (31).
‡ Reciprocal of the highest dilution of mouse serum at which borrelia growth is inhibited in culture (34).
§ Vector used to express OspA gene in rBCG vaccine.
‖ Nonrecombinant BCG (Pasteur substrain).

ficient mouse models for Lyme borreliosis, and this protection has been shown to be antibody mediated (27–29). Immune sera collected 2 wk after a single booster immunization of C3H and BALB/c mice immunized with the different rBCG-OspA vaccines were analyzed for their ability to inhibit growth of the culture-adapted B31 laboratory strain of *B. burgdorferi* in vitro (34). Antisera derived from immunizations with both rBCG-L-OspA vaccines (rBCG-p19PS::*ospA*, rBCG-pMV251::*ospA*) exhibited high-titered *B. burgdorferi* growth-inhibiting activity, while sera derived from mice immunized with the rBCG-C-OspA vaccine (rBCG-pMV261::*ospA*) or nonrecombinant BCG showed low or undetectable growth-inhibiting titers (Table 2). In identical assays, immune sera from rBCG-OspA-immunized mice failed to inhibit the growth of *B. hermsii*, which does not express OspA (data not shown). This demonstrated that growth inhibition was OspA specific and not due to a nonspecific mode of action against borrelias.

Based on these results, C3H and BALB/c mice were challenged intraperitoneally with 10^6 borrelia spirochetes derived from an infectious clone of the low-passage Sh.2 strain of *B. burgdorferi* (35) of the same OspA serogroup as B31 (47).

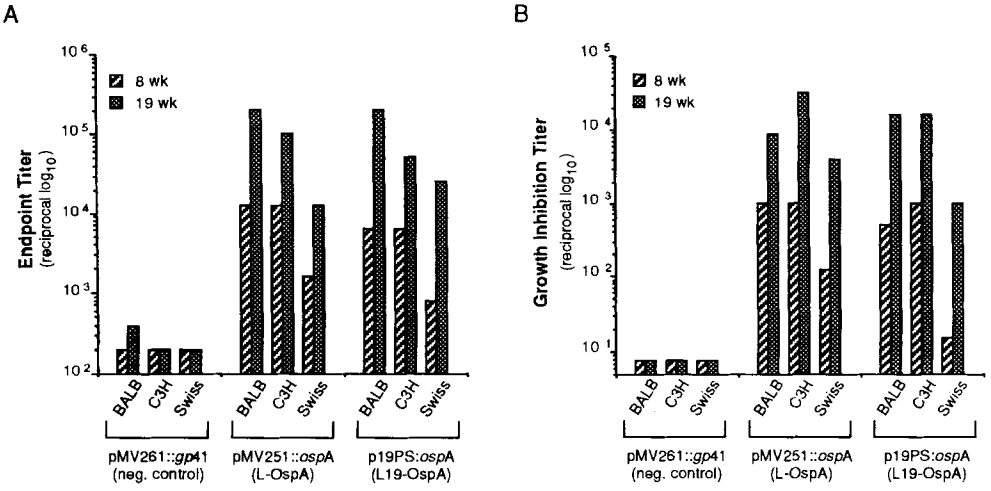

Figure 7. Immunogenicity of rBCG L-*ospA* constructs. The indicated mouse strains were immunized by the intraperitoneal route with 10^6 CFU of the indicated rBCG and boosted at 17 wk with an identical dose. Sera were collected at the indicated times from each group (five mice), pooled, and analyzed by ELISA with whole *B. burgdorferi* (*A*) and in the in vitro growth inhibition assay (*B*) (34).

The ability of rBCG-OspA-immunized mice to inhibit *B. burgdorferi* growth in vivo was assayed by culture of bladder and heart tissues taken from mice 2 wk after challenge, the normal peak of spirochetemia in a borrelia-infected mouse. All mice immunized with an irrelevant BCG control were found to be infected, as determined by positive borrelia culture from bladder tissue. Heart cultures appeared to be a less-sensitive indication of infection in these control mice. Three of four groups of mice immunized with rBCG-OspA lipoprotein vaccines were completely protected from intraperitoneal challenge with 10^6 spirochetes, as determined by bladder culture of borrelia (Table 2). Evidence of borrelia breakthrough was observed with this high-challenge dose in one group of C3H mice immunized with the rBCG-p19PS vaccine despite substantial neutralizing antibody titers. However, this rBCG L-OspA vaccine completely protected BALB/c mice. In contrast, immunization with rBCG vaccines expressing OspA as a cytoplasmic protein, or as a secreted/cell wall–associated protein, resulted in incomplete protection against challenge. Some reduction in culturable borrelia from heart tissue was observed, but no significant differences were found in the culture of the bladder tissues compared with control BCG-immunized mice (Table 2).

It has been shown that the intradermal route of borrelia challenge is more efficient for establishing infection in mice and is presumably more relevant to an infection delivered by a tick bite (48). Therefore, in a second experiment we determined whether the rBCG-L-OspA vaccines eliciting the highest antibody titers and protective responses against intraperitoneal borrelia challenge (rBCG-pMV251::*osp*A and rBCG-p19PS::*osp*A) could similarly protect mice against intradermal challenge. As in the first experiment, C3H, BALB/c, and Swiss mice were immunized intraperitoneally with 10^6 CFU of each rBCG vaccine, and boosted at 17 wk. All three strains of mice immunized with rBCG-L-OspA vaccines again exhibited high OspA-specific antibody titers before and after boost, while mice immunized and boosted with an irrelevant BCG control did not (Fig. 7 *A*). Immune sera collected from C3H and BALB/c mice 8 wk after the primary immunization with rBCG-L-OspA vaccines exhibited substantial growth-inhibiting antibody titers (1:500–1,000), while sera from all three mouse strains collected 2 wk postboost exhibited higher growth-inhibiting antibody titers (Fig. 7 *B*). All immunization groups were challenged intradermally 4 wk postboost with 10^4 borrelia, and plasma, bladder tissue, and tibiotarsal joints were cultured for detection of borrelia infection 14 d postchallenge. Both rBCG-L-OspA vaccines completely protected all three mouse strains against intradermal challenge; spirochetes could not be cultured from any of the tissues or plasma tested (Table 3). In contrast, all mice immunized with a control irrelevant recombinant BCG were shown to be infected, as indicated by the positive culture of *B. burgdorferi* spirochetes from either plasma, bladder, or joint tissues. The 10^4 borrelia challenge dose used in this intradermal challenge experiment is roughly estimated to be comparable to an infection from 5–50 infected flat nymphal stage ticks (*Ixodes dammini*) (49).

Discussion

Significant progress has been made toward the identification of protective antigens for a wide variety of diseases but basic difficulties central to practical vaccine development still exist. Among the most formidable obstacles is the cost-effective production of immunogenic vaccine components in sufficient quantities for wide use and the delivery of these components

Table 3. *Protection against Intradermal Challenge with B. burgdorferi (Exp. 2)*

rBCG vector§ immunogen	In vivo protection:* no. mice with culturable borrelia in tissues or blood/total no. mice											
	Bladder culture			Blood culture			Joint culture			Total culture positive‡		
	BALB	C3H	Swiss	BALB	C3H	Swiss	BALB	C3H	Swiss	BALB	C3H	Swiss
pMV251::*osp*A (L-OspA)	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
p19PS::*osp*A (L19-OspA)	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
pMV261::gp41‖ (rBCG control)	4/4	4/5	2/5	4/4	4/5	4/4	3/4	4/5	2/4	4/4	5/5	4/4

100 LD_{50} (10^4 spirochetes) of low-passage Sh.2 strain (31, 35).
* Number of mice in each group testing positive for borrelia infection by culture of tissues 14 d after challenge as a fraction of the total number of mice per group (31).
‡ Number of mice culture positive for any single tissue or blood culture.
§ Vector used to express OspA gene in rBCG vaccine.
‖ rBCG expressing HIV-1 gp41 in expression vector pMV261 (1).

in a safe and immunogenic form. One approach taken to overcome vaccine production and delivery problems is the development of live attenuated vaccines that replicate and express protective antigens in vivo. With the development of recombinant DNA methodology has come approaches to modify live attenuated vaccines, (e.g., pox viruses and *Salmonella*) to produce heterologous antigens protective against a variety of diseases. As a widely used human vaccine against tuberculosis, the live attenuated BCG vaccine also offers considerable advantages for development as a multivalent vaccine vehicle for other human pathogens (4). With the recent development of genetic vector systems for the slow-growing mycobacteria, it is now possible to evaluate the potential for recombinant BCG as a multivalent live carrier for inducing protective immunity to heterologous antigens (1–5).

Inasmuch as BCG is rapidly ingested by macrophages and grows within the macrophage phagolysosome, rBCG is usually thought of as a vaccine vehicle for eliciting stronger cellular responses than humoral responses. Initial studies with model rBCG vaccines expressing *E. coli* β-galactosidase indicated that substantial cellular and humoral responses to target antigens delivered as rBCG cytoplasmic proteins were possible (1). Subsequent follow-up studies with a number of target antigens expressed in the cytoplasm of rBCG have indicated, however, that primary target antigen–specific antibody responses in mice are generally more difficult to obtain or are highly variable and strain specific. To improve the potential of recombinant BCG vaccines to elicit protective humoral responses to target antigens, we have developed expression vectors using signal sequences derived from mycobacterial lipoproteins to direct the export of recombinant target antigens to the membrane of the BCG carrier as immunogenic chimeric lipoproteins. This strategy was based on the reasoning that bacterial lipoproteins are membrane associated, sometimes located at the surface of the bacterium, and are often among the most immunogenic antigens recognized by the infected host's humoral immune response (15–22). In the case of *M. tuberculosis*, at least two lipoproteins, the 19- and 38-kD (Mtb19 and Mtb38) antigens, are among the most immunogenic of antigens recognized by infected humans (37, 38). While the immunogenic nature of some lipoproteins may be due in part to their location in the membranes of bacteria, evidence is mounting that the inherent immunogenicity of lipoproteins is due in large part to the NH_2-terminal lipid moiety. This has now been directly demonstrated for OspA (31, and this study). Furthermore, chemical lipid acylation of peptides and proteins can profoundly enhance their immunogenicity (12, 13). A definitive explanation for this widely observed augmentation of immunogenicity is not yet available, but as free lipoproteins or lipopeptides, it might be possible that the NH_2-terminal lipid moieties might associate as micelles, which could be more readily taken up by APCs. This could then enhance antigen presentation to T cells, which could in turn result in improved T help for cytotoxic cellular responses and B cell humoral responses. While this explanation might be tenable for subunit vaccines it does not explain why a lipoprotein expressed on rBCG, which is readily taken up by APCs, might be more immunogenic than the same antigen expressed in the cytoplasm of BCG. It is perhaps possible that exterior lipoproteins of rBCG are shed and are therefore more rapidly available to antigen processing mechanisms in the macrophage phagolysosome than are BCG cytoplasmic proteins. It might also be possible that L-OspA expressed on the surface of rBCG can directly prime B cells when BCG are extracellular, resulting in a synergistic response that normally does not occur to the same antigen expressed in the cytoplasm of BCG. Yet other explanations might be based on alternative antigen processing pathways for lipoproteins or the possibility that some lipoproteins or lipopeptides are mitogenic (12, 14). It should be noted, however, that enhanced humoral immune responses to BCG antigens were not observed in mice immunized with rBCG L-OspA vaccines (data not shown), arguing against the possibility that L-OspA expression in rBCG is somehow mitogenic. The elucidation of the mechanism by which lipid acylation enhances OspA reactivity requires further study. Such studies should include a comparison of T cell responses to OspA and additional antigens expressed cytoplasmically or extracellularly as lipoproteins or secreted proteins.

The insoluble nature of lipoproteins makes them generally more difficult to evaluate as candidate vaccines (e.g., OspA; 30). It is also likely that some lipoproteins require membrane association to exhibit authentic conformational epitopes. The expression of target lipoprotein antigens in rBCG obviates the need to purify the membrane-bound and insoluble lipoproteins, and presents the opportunity to express these antigens in a more authentic membrane-associated context. In addition to the delivery of natural lipoproteins by rBCG, it may be possible to enhance the immunogenicity of nonlipoprotein antigens by making them chimeric lipoproteins fused to mycobacterial signal peptides. While many target antigens will be refractory to export and lipid acylation by rBCG, additional studies in this laboratory indicate that some nonlipoprotein antigens can also be exported and lipid modified with the Mtb19 and Mtb38 lipoprotein (50) signal sequences using the same LPE vectors described in this study (e.g., PspA of *Streptococcus pneumoniae*; Stover et al., manuscript in preparation). Studies are in progress to determine whether the immunogenicity of nonlipoprotein target antigens can be enhanced by rBCG delivery as chimeric membrane-associated lipoproteins. In comparison with other approaches involving the grafting of epitopes into bacterial cell surface structures, the chimeric lipoprotein approach used in this study has several advantages: (*a*) only minimal sequence information may be necessary to direct export of recombinant target antigens; (*b*) the signal peptide directing export is ultimately removed, minimizing contextual effects on important antigenic determinants; and (*c*) the target antigen is membrane anchored by means of an NH_2-terminal lipid moiety that may enhance immunogenicity (11–14). Since BCG has a single membrane, it may also be generally easier to export foreign antigens from rBCG than it is in Gram-negative bacterial vector systems with inner and outer membranes separated by a periplasmic space.

By all immunological and functional criteria measured, rBCG vaccines expressing OspA as a lipoprotein were superior to rBCG vaccines that express OspA in their cytoplasm or as a secreted/surface protein associated, at least in part, with the BCG cell wall. rBCG-OspA lipoprotein vaccines elicited earlier, higher titered, protective anti-OspA responses that were consistent from mouse to mouse. These protective humoral responses were also induced in mice that are lower responders to OspA. The comparison of anti-OspA responses generated by rBCG expressing L-OspA and L19-OspA with the responses generated by rBCG expressing secreted surface-expressed S-OspA suggests that lipid acylation of OspA may have a greater impact on the potential to generate humoral responses to OspA than surface expression by BCG. However, a strict comparison is not possible with these data as it was necessary to fuse OspA to a full-length secreted protein (αAg) to obtain a secreted/cell wall–associated fusion protein. It is of interest that rBCG expressing potentially authentic OspA polypeptide encoded by plasmid pMV251::*ospA* exhibited surface fluorescence and elicited a high-titered and protective anti-OspA response even though only a very small amount of the protein appears to be authentically processed (lipid-modified L-OspA) in comparison with rBCG vaccine expressing L19-OspA from p19PS::*ospA*. This may occur because the borrelia OspA signal peptide is sufficiently dissimilar from that recognized by mycobacterial acylation enzymes, so that this lipid modification, a prerequisite for signal peptidase II–mediated export, occurs very inefficiently. The nonacylated OspA might then be exported by the major signal peptidase I–mediated pathway. However, this explanation would also require OspA to be anchored to the exterior of BCG in the absence of lipid modification (OspA protein was never found in rBCG-pMV251::OspA culture supernatants) and would also suggest that BCG surface-expressed nonlipid acylated OspA is very immunogenic. These explanations seem unlikely in light of the recent evidence that purified free OspA lipoprotein is profoundly more immunogenic than nonlipid acylated OspA (31). Rather, it is more likely that the BCG-pMV251::OspA vaccine is very effective because only a very small amount of L-OspA in the context of rBCG is actually necessary to achieve enhanced anti-OspA immune responses. This small amount of authentic L-OspA expressed in rBCG-pMV251::*ospA* may be more immunogenic per molecule equivalent simply because it is more authentic than the lipid-modified chimeric L19-OspA, which carries an additional five amino acid residues from the Mtb19 lipoprotein as its NH_2 terminus. It is also of interest that rBCG expressing OspA as a membrane-associated lipoprotein are attenuated in their ability to replicate and persist in vivo as compared with nonrecombinant BCG (data not shown). This is in contrast to what one would expect for a live vaccine, which elicits enhanced target antigen–specific immune responses. The effect of this attenuation on the ability of the rBCG vaccine to elicit cell-mediated immunity and to protect against *M. tuberculosis* challenge is currently under investigation in the mouse model.

Although comparisons between immune responses elicited by OspA lipoprotein subunit vaccines with live rBCG-OspA vaccines were not the aim of this study, comparisons with a recently published study on a purified L-OspA subunit vaccine (31) suggest advantages for the delivery of OspA lipoprotein by rBCG. Comparison of growth inhibition titers obtained from both studies is possible as these titers were obtained from the same mouse strain sera (BALB/c and C3H) by identical assays in the same laboratory (Sadziene, A., unpublished data). Primary immunization and boosting of C3H mice and BALB/c mice with the highest doses tested (10 and 2.5 μg, respectively) resulted in maximum growth inhibition titers of 1:1,024 and 1:512 in the previous study on the L-OspA subunit vaccine (31). In experiment 1 of the present study, immunization and boosting of the same mouse strains with either rBCG L-OspA vaccine resulted in borrelia growth-inhibiting titers ranging from 1:1,024 to 1:4,096. Higher growth-inhibiting titers (1:8,924–32,768) were obtained in C3H and BALB/c mice in experiment 2. Moreover, immunization of three different mouse strains with a single dose of rBCG L-OspA and L19-OspA vaccines resulted in substantial anti-OspA titers ranging from $1:10^3$ in the outbred Swiss Webster mouse to $1:10^4$ in the inbred C3H and BALB/c mice. These primary responses, which lasted in >16 wk, resulted from immunization with a rBCG inocula of 10^6 CFU that initially contained only 1–5 ng of L-OspA. In the previous study, immunization with 10 μg of purified L-OspA (200–1,000-fold more L-OspA than delivered in the initial rBCG L-OspA inoculum) elicited very weak but detectable primary responses in the more OspA-responsive C3H mice, while primary responses in the less OspA-responsive BALB/c mice were not reported (31). These observations suggest the enhancement of OspA immunogenicity by delivery as a membrane-associated lipoprotein on the live rBCG vaccine vehicle. It is possible that this is the result of adjuvant effects provided by the BCG cell wall, and/or a persistent "slow release" of OspA antigen by the live rBCG vaccine, which may continually restimulate the immune response as the organism persists or grows in the host.

These studies have provided the first demonstration of protection elicited by a recombinant BCG vaccine and the development of a promising candidate vaccine for the immunoprophylaxis of Lyme disease. Delivery of OspA as a lipoprotein in the live replicating rBCG vector not only obviates the need to purify and formulate sufficient quantities of an OspA subunit vaccine, but rBCG delivery may also elicit more uniform responses in vaccinees of a heterogeneous genetic background. Further studies are necessary to determine whether the immunogenicity of other lipoprotein and nonlipoprotein target antigens can be enhanced by delivery as chimeric membrane-associated lipoproteins on rBCG. We are also investigating whether this approach will also enhance cellular immune responses to target antigens delivered by rBCG. Such studies could illuminate the mechanism by which lipid modification or membrane-associated expression can augment antigen immunogenicity.

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