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# Outer Membrane Protein of *Neisseria meningitidis* as a Mucosal Adjuvant for Lipopolysaccharide of *Brucella melitensis* in Mouse and Guinea Pig Intranasal Immunization Models

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**A mucosal vaccine against brucellosis consisting of the lipopolysaccharide (LPS) of *Brucella melitensis* complexed with the outer membrane protein (GBOMP) of group B *Neisseria meningitidis* was tested in small-animal models of intranasal immunization. Mice given two doses of the vaccine developed high levels of immunoglobulin G (IgG) and IgA antibodies specific for *B. melitensis* LPS in lung lavages and specific IgG and IgA antibody-secreting cells in the lungs and spleen. Similarly, in guinea pigs immunized twice intranasally, IgG and IgA LPS-specific antibodies were detected in lung lavages, and specific antibody-secreting cells were isolated from the spleen and cervical nodes. In mice immunized with LPS only, pulmonary responses consisted mostly of IgM antibodies, while guinea pigs given LPS alone developed local antibody of all three isotypes, but at lower levels compared to animals given the complex vaccine. Both mice and guinea pigs also developed high levels of serum IgG and moderate levels of IgA as a result of intranasal immunization with the complex vaccine. The serum antibodies in both cases were found to cross-react with the LPS of *B. abortus*, which shares an immunogenic epitope with *B. melitensis* LPS. In mice given the complex vaccine, there was a prominent serum IgG1 response that was absent in the mice given LPS alone. In conclusion, the *N. meningitidis* GBOMP was an effective mucosal adjuvant for secretory IgA and IgG responses in the lungs of both mice and guinea pigs. The IgG1 subclass response in mice suggests that GBOMP may have favored a $Th_2$ type of response to the LPS. A vaccine capable of stimulating high levels of antibody at local sites has the potential to protect against *brucellae*, since these pathogens gain entry to the host via mucosal routes.**

Brucellae are gram-negative intracellular pathogens which cause zoonotic disease in humans. Typically, infection is initiated when the bacteria gain entry at a mucosal site such as the conjunctiva, the respiratory or intestinal tract, or through abrasions in the skin (17). In animals, brucellae preferentially localize to reproductive organs and to fetal tissue, thereby causing abortion, while in the human host they tend to proliferate in the reticuloendothelial system. In addition to early symptoms such as fever, chills, and weakness, brucellae can survive for long periods within macrophages and cause chronically relapsing disease (2). Cellular immune responses are expected to play an important role in eradication or containment of intracellular infections such as those caused by brucellae (16, 18, 25). Systemic lipopolysaccharide (LPS)-specific antibodies have also been shown to be protective (24). Other investigators have shown that secreted immunoglobulin A (IgA) can protect mice against bacteria (20) and viruses (7) in the respiratory tract. Whether secretory antibodies can protect against brucellosis is not known.

To optimize the mucosal IgA response, various adjuvants such as cholera toxin (15) and *Escherichia coli* labile toxin (3) and carrier systems such as liposomes (1) have been developed. *Neisseria meningitidis* group B outer membrane protein (GBOMP) has been used previously as a carrier for protein and polysaccharide immunogens in parenteral vaccination regimens (14, 27) and for construction of proteosome-LPS vaccines for mucosal delivery (19). In this study, we investigated the ability of *N. meningitidis* GBOMP to act as a mucosal adjuvant for the delivery of *Brucella melitensis* LPS in small-animal immunization models. *N. meningitidis* GBOMP and *B. melitensis* LPS were combined to form a complex for intranasal vaccination of mice and guinea pigs. The ultrastructure of the complex was examined by electron microscopy. Local and systemic antibody responses were measured following immunization with *B. melitensis* LPS alone or with the LPS-GBOMP complex. Antibody-secreting cell (ASC) responses were also measured in order to study isotype distribution and trafficking of antigen-specific B cells following induction in the lung mucosa. Differences in IgG subclass responses were examined in the mice as a possible reflection of differences in T helper cell activity.

## MATERIALS AND METHODS

**Preparation of purified LPS.** Purified LPS was purified by a slight modification of the method of Bundle et al. (6). Bacteria were grown in Brucella broth (Difco, Detroit, Mich.) for 48 h at 37°C in a biosafety level 3 laboratory. Cells from a 10-liter culture were suspended in 1-liter extraction buffer (0.1% Tris-HCl [pH 7.2] containing 1% NaCl and 2% phenol) and stirred at 50°C for 5 days. Bacteria were pelleted by centrifugation at 7,000 × *g* for 20 min. The supernatant was removed and dialyzed extensively against distilled water to remove the phenol and was then concentrated by ultrafiltration on a PM-10 membrane (Amicon Corporation, Boston, Mass.) to half the original volume. The concentrate was

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centrifuged at 105,000 × *g* for 5 h, and the resultant pellets were resuspended in water and lyophilized to obtain crude LPS. Crude LPS was extracted twice with chloroform-methanol (2:1) and then partitioned between chloroform and water. The water phase was lyophilized to obtain partially purified LPS, which was digested with DNase, RNase, and proteinase K and was again centrifuged at 105,000 × *g* for 5 h. Resultant pellets were lyophilized to obtain purified LPS, which contained <2% nucleic acid as determined by absorbance at 260 and 280 nm and <5% protein as determined by the BCA method (21). The yield of this procedure was 2 to 3 mg of purified LPS per liter of culture.

**Production of the LPS-OMP complex vaccine.** Purified *B. melitensis* 16M LPS was noncovalently complexed with *N. meningitidis* GBOMP as previously described (4). Nine milligrams of LPS was dissolved in 2.2 ml of sterile saline (0.9% NaCl). GBOMP (5.8 ml; 1.6 mg/ml) in 0.1% TEEN buffer (0.05 M Tris, 0.01 M EDTA, 0.15 M NaCl, 0.1% Empigen BB [Albright & Wilson, Ltd., Cumbria, England] [pH 8.0]) was added with stirring to the LPS solution. The mixture was placed into dialysis tubing (12,000 molecular weight cutoff) and dialyzed in 500 ml of sterile saline at 5°C without stirring for 5 h and then with stirring for 3 days, followed by 2 cycles of 500 ml of fresh saline and stirring for 2 days. The resultant vaccine, in a volume of 8 ml, was filtered through a 0.22-μm membrane and stored at 5°C. Protein content (1.08 mg/ml) was determined by the BCA method, and LPS content (970 μg/ml) was determined by the phenol-sulfuric acid method (12).

**Electron microscopy studies.** LPS-GBOMP vaccine preparation was evaluated by electron microscopy. Fifteen microliters of each sample was applied to a carbon- and Formvar-coated 300-mesh copper grid for 1 min. Excess liquid was removed, and the sample was immediately stained with 15 μl of 0.5% uranyl acetate and 0.5% trehalose, pH 4.5. After 30 s, the stain was completely removed and the specimen was allowed to air dry. The sample was examined with a Philips 400 transmission electron microscope operating at an accelerating voltage of 80 kV.

**Immunization of animals.** Animals were immunized by the intranasal route under light anesthesia by intramuscular injection of a mixture of 0.3 mg of xylazine hydrochloride (Rompun; Mobay Corp., Shawnee, Kans.) and 1.0 mg of ketamine hydrochloride (Ketaset; Aveco Co., Fort Dodge, Iowa) in 50 μl of saline for mice or 1.7 mg of xylazine hydrochloride plus 16.7 mg of ketamine hydrochloride in a volume of 250 μl for guinea pigs. Mice were routinely given 10 μg of LPS (alone or as an LPS-GBOMP complex), and guinea pigs were given 50 μg, in volumes of 30 and 100 μl, respectively, dropwise onto the nares. Following the initial immunization, mice were boosted on day 29 and guinea pigs were boosted on day 14. All animal experiments were repeated at least once.

**Sampling of immune response.** Mice were euthanized by $CO_2$ inhalation, and guinea pigs were evaluated by injection of phenobarbital. Blood was then obtained by cardiac puncture, and sera were stored at −20°C. Lung lavages for mice were performed by inflating the lungs with 0.8 ml of RPMI 1640 supplemented with 50 μg of gentamicin (Gibco, Gaithersburg, Md.) per ml via the trachea by using a 1-ml syringe fitted with a 24-gauge catheter (Critikon, Tampa, Fla.), and guinea pig lungs were lavaged by inflation with 3 ml of RPMI 1640 with a syringe fitted with a 21-gauge needle. Fluids withdrawn from inflated lungs were centrifuged to remove debris, and the supernatants were stored at −70°C until assayed. Mononuclear cells (MNC) were harvested from lungs and spleens of mice and from cervical nodes and spleens of guinea pigs for use in the ELISPOT (10) assay for ASC enumeration. MNC were isolated by passage of minced tissues through wire mesh screens. Cell suspensions were then treated with ammonium chloride (Sigma, St. Louis, Mo.) to lyse erythrocytes, and remaining cells were washed, counted, and resuspended in culture medium consisting of RPMI 1640 supplemented with 10% fetal calf serum, 2 mM L-glutamine, and 50 μg of gentamicin per ml (all Gibco) at a density of $2.5 \times 10^6$/ml.

**ASC.** Previously described procedures for detection of ASC responses in mice (23) and guinea pigs (13) were followed. Prepared MNC suspensions were dispensed in 100-μl volumes into quadruplicate flat-bottom wells of microtiter plates (Nunc, Roskilde, Denmark) previously coated with 1 μg of LPS in phosphate-buffered saline (PBS) or with PBS alone. Plates were incubated for 4 h at 37°C. For mouse studies, wells were then washed and to each was added goat anti-mouse IgG, IgA, or IgM antiserum conjugated to alkaline phosphatase (Kirkegaard-Perry, Gaithersburg, Md.). Plates were incubated overnight at 4°C and washed, and to each well was added 100 μl of molten (60°C) agarose containing 100 μg of 5-bromo-4-chloro-3-indolyl phosphate per ml (all Sigma). For guinea pig studies, after the incubation step with cells, wells were washed and unconjugated rabbit anti-guinea pig IgG, IgA, or IgM (ICN Pharmaceuticals, Costa Mesa, Calif.) was added. Overnight incubation at 4°C was followed by washing and addition of alkaline phosphatase-conjugated goat anti-rabbit antisera (Sigma), incubation for 2 h at 37°C, and then addition of an agarose-substrate mix as for mouse studies. Spot-forming cells (B-cell blasts actively secreting specific antibodies) were counted with a stereomicroscope and recorded as the number of ASC/$10^6$ MNC.

**ELISA.** Alternating columns of polyvinyl microtiter wells were coated with 50 μl of LPS (10 μg/ml) in PBS (test wells) and with 50 μl of PBS alone (blank wells) for mouse studies; wells for guinea pig studies were coated likewise with 100-μl volumes. Sera were serially diluted in paired columns, beginning with 1:20 for mice and 1:50 for guinea pigs. Lung lavages were tested at single dilutions (1:4 for mice and 1:2 for guinea pigs) in paired test and blank wells. Plates were incubated overnight at 4°C and washed, and bound antibodies were detected by the addition of antisera as described above for ASC. Incubations were for 2 h at 37°C for each antiserum step. For mouse IgG subclass studies, overnight incubation at 4°C with primary antibody was followed by rabbit anti-mouse IgG1 or IgG2a (ICN) and then goat anti-rabbit IgG conjugated to alkaline phosphatase (Sigma) in 2-h incubation steps as for other ELISAs above. Following the final washing step, 100 μl of the pNP substrate (Kirkegaard-Perry) was added to all wells and optical density (OD) was measured at 410/570 nm. Blank well values were subtracted from corresponding test well values to yield net OD. For serially diluted specimens, endpoint titers were defined as the highest dilution with net OD of ≥0.100. Statistical analysis was performed by the Wilcoxon rank sum test.

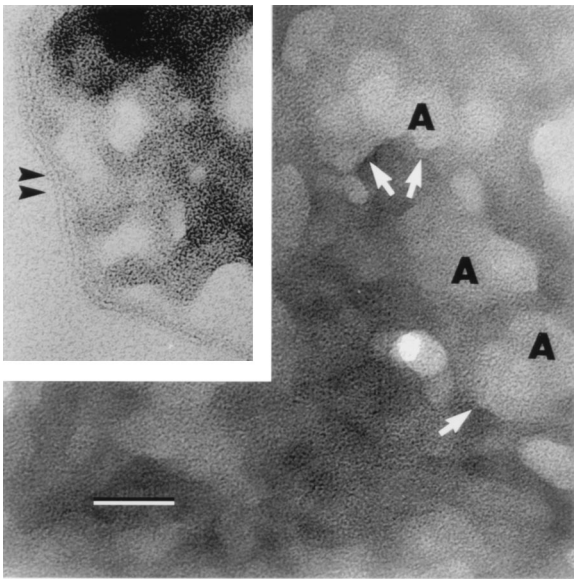


FIG. 1. Electron photomicrograph of negatively stained LPS-GBOMP vaccine preparation showing two distinct morphological structures: aggregated outer membrane vesicles (A), some of which formed amorphous stacked assemblages (white arrows), and outer membrane constituents coalesced into bundles (inset, arrowheads) of three or more linearized fragments. The specimen was stained with 0.5% uranyl acetate–0.5% trehalose, pH 4.5. Bar, 50 nm.

## RESULTS

**Ultrastructure of the vaccine.** Uranyl acetate-stained preparations of the vaccine (Fig. 1) showed that it consisted of two distinct morphological structures. The first and most abundant type of structure observed was aggregated outer membrane vesicles of <50 nm, some of which formed amorphous stacked assemblages. The second type of structure observed was linearized membrane fragments. These observations are consistent with earlier studies of native outer membrane complexes of *N. meningitidis*, which demonstrated predominantly closed structures of variable size and morphology bounded by a single trilaminar membrane (26).

**Responses of mice.** *B. melitensis* LPS-specific ASC were measured in mouse lungs to assess local B-cell responses to immunization. Splenic ASC were also measured as a reflection of the migration of activated B cells to distal sites. Additionally, it is possible that antigen may have been transported to the spleen to initiate an immune response at that site. In mice immunized once with LPS-GBOMP, ASC were detectable in spleen (Fig. 2A) and lung (Fig. 2C) MNC as early as day 7; ASC were predominantly of the IgM isotype in the spleen and IgA in the lungs. Spleen IgM ASC peaked on day 7 and declined to less than 10/$10^6$ MNC by day 28. IgA ASC in the lungs peaked on day 14 and were essentially undetectable by day 28. Antibody isotype distributions of lung and spleen ASC were more similar after the second immunization on day 29, which stimulated a dramatic rise in LPS-specific IgA ASC at

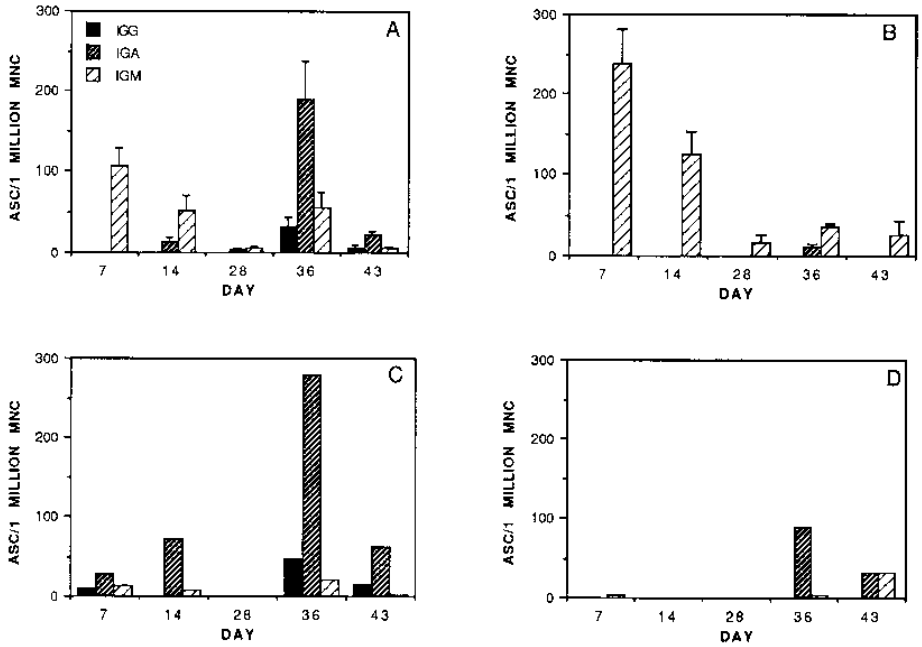


FIG. 2. Time course of *B. melitensis* LPS-specific ASC responses of mice immunized with LPS-GBOMP (A and C [spleen and lungs, respectively]) or with LPS alone (B and D [spleen and lungs, respectively]). Mice were immunized on days 0 and 29 of the experiment. Each data point in panels A and B is the mean of values from three animals; error bars represent 1 standard error. In panels C and D, each data point represents a result of pooling cells from three animals.

both sites by day 36. Rises in IgG ASC above levels seen after the first immunization were also detected at both sites. In contrast, mice given LPS alone developed LPS-specific IgM ASC at high levels in the spleen (Fig. 2B) at 7 days after primary immunization; this response dropped gradually over the course of the study and was not boosted by the second immunization. A small number of IgA ASC was detected at day 36 (mean, $<25/10^6$ MNC; $P = 0.05$ compared to LPS-GBOMP-treated mice). In the lungs (Fig. 2D), this group of mice had less than 10 IgM ASC/$10^6$ MNC on day 7, and levels of these cells were not increased after secondary immunization. A moderate rise in numbers of IgA ASC (less than 100/$10^6$ MNC) was seen at 7 and 14 days after the second immunization. The specificity of the ASC assay was confirmed by the lack of any ASC in wells coated with PBS.

*B. melitensis* LPS-specific antibody levels in the lungs of mice are summarized in Fig. 3. In animals given the complex vaccine (Fig. 3A), IgA was the predominant isotype in the lung lavages and was detectable by 14 days after primary immunization. IgA levels continued to rise throughout the study with a marked increase seen at 7 days after the boost (day 36), reflecting the expansion of LPS-specific IgA ASC in the lungs. LPS-specific IgG levels were detected at 28 days after primary immunization, and a marked increase was also seen as a result of boosting. IgM levels rose after the boost, but, unlike IgA and IgG, levels began to wane by day 43.

In mice immunized with LPS alone (Fig. 3B), a low level of LPS-specific IgM only was seen at 28 days; these antibodies were not increased after the second immunization. Although there was a moderate rise in local IgA at 7 days after boosting ($P = 0.05$ compared to LPS-GBOMP-treated mice), these antibodies were greatly reduced a week later. In marked contrast to mice immunized with LPS-GBOMP, no local IgG was detected in the lungs of this group of mice vaccinated with purified LPS.

In general, peak pulmonary antibody levels occurred about a week behind peak ASC levels. This may due to the accumulation and persistence of local antibodies after the activated B cells have died off. Mice immunized in parallel with saline or GBOMP alone were all negative for antibody responses to *B. melitensis* LPS (data not shown).

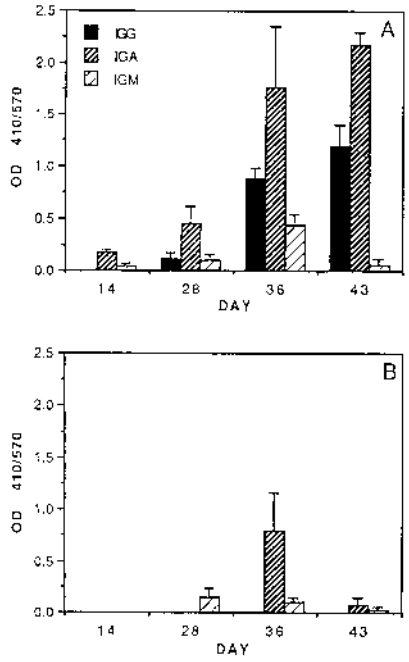


FIG. 3. Time course of *B. melitensis* LPS-specific pulmonary antibody development in mice immunized with LPS-GBOMP (A) or with LPS alone (B). Each data point is the mean of values from three animals; error bars represent 1 standard error.

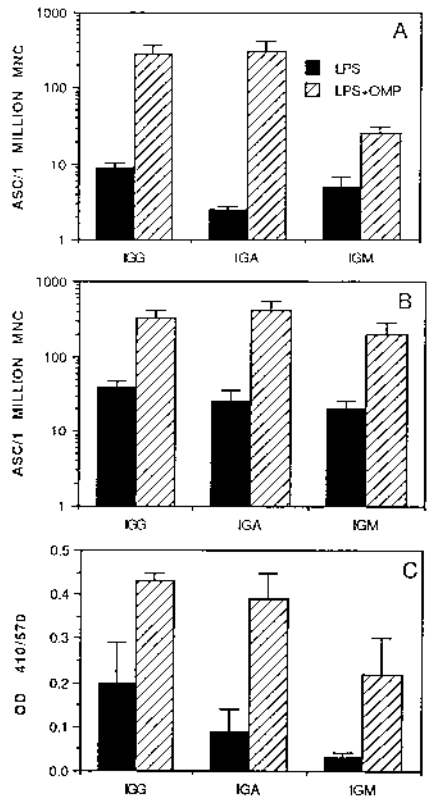


FIG. 4. *B. melitensis* LPS-specific ASC responses detected in the spleen (A) and cervical nodes (B) of guinea pigs at 7 days after boosting. Levels of anti-*B. melitensis* LPS pulmonary antibodies from the same animals are shown in panel C. Each data point is the mean of values from four animals; error bars represent 1 standard error.

**Responses of guinea pigs.** Guinea pigs immunized twice with LPS-GBOMP had significantly more *B. melitensis* LPS-specific ASC of IgG, IgA, and IgM isotypes in the cervical nodes which drain the head region (Fig. 4A) when compared with animals given LPS alone. IgA- and IgG-secreting cells were predominant and were detected at equal levels. Similar ASC responses were seen in the spleens of these animals at the same time (Fig. 4B). In parallel, specific antibody responses of similar isotype distributions were detected in lung lavage fluids (Fig. 4C). Unlike the case with mice, guinea pigs given LPS alone were able to develop LPS-specific ASC responses of all three isotypes at both the local (cervical node) and distal (spleen) sites; however, ASC responses were at least 10-fold lower than those of animals given the LPS-GBOMP vaccine ($P < 0.05$ for both cervical node and spleen ASC). Pulmonary antibody levels in guinea pigs immunized with LPS were also significantly reduced compared to animals given LPS-GBOMP ($P < 0.05$).

**Serum antibodies.** LPS-specific serum antibody responses were measured to determine the effect of intranasal immunization on humoral antibody levels. Serum antibody responses (expressed as geometric mean titers) of mice at 28 days after primary immunization and at 14 days after secondary immunization and of guinea pigs at 7 days after secondary immunization are summarized in Table 1. LPS-GBOMP-treated mice and guinea pigs developed serum antibody responses of all three isotypes to *B. melitensis* LPS; these antibodies included a subpopulation which cross-reacted with the LPS of *B. abortus*. In the mice, the first immunization with LPS-GBOMP stimulated higher levels of IgG and IgA but not IgM compared to immunization with LPS alone ($P = 0.05$). The second immunization boosted IgG and IgA titers about 16-fold above levels seen after primary immunization, while IgM levels were increased less than 2-fold. Mice given LPS alone did not develop any serum IgA throughout the study; boosting with LPS caused only a modest rise in IgG ($P = 0.05$ compared to LPS-GBOMP-treated mice) but not IgM levels. Guinea pigs given LPS did develop serum antibodies of all three isotypes, but, as with ASC responses, the magnitudes were much lower compared to those of LPS-GBOMP-treated animals ($P < 0.05$).

TABLE 1. Mouse and guinea pig serum antibody responses

| Treatment | Geometric mean postimmunization titer to LPS | | | | | |
|---|---|---|---|---|---|---|
| | *B. melitensis* | | | *B. abortus* | | |
| | IgG | IgA | IgM | IgG | IgA | IgM |
| Mice ($n = 3$) | | | | | | |
| LPS | | | | | | |
| Immunized once | 160 | ND[a] | 508 | 16 | ND | 640 |
| Immunized twice | 806 | ND | 403 | 25 | ND | 202 |
| LPS-GBOMP | | | | | | |
| Immunized once | 1,613 | 63 | 254 | 127 | 40 | 403 |
| Immunized twice | 25,807 | 1,016 | 403 | 12,902 | 320 | 202 |
| Guinea pigs[b] ($n = 4$) | | | | | | |
| LPS | 2,263 | 71 | 476 | 1,345 | 42 | 336 |
| LPS-GBOMP | 121,775 | 4,526 | 3,805 | 102,400 | 2,690 | 1,600 |

[a] ND, none detected.
[b] Immunized twice.

In repeated experiments, both mice and guinea pigs immunized with LPS-GBOMP consistently developed higher ASC and local and humoral antibody responses compared to animals immunized with LPS alone.

**IgG subclasses in mice.** IgG subclass responses in mice are known to be affected by the action of T helper (Th) cell subsets (22). IgG1 and IgG2a responses (favored by Th2 and Th1 subsets, respectively) to *B. melitensis* LPS are summarized in Fig. 5. IgG1 responses (Fig. 5A) were seen in both LPS- and LPS-GBOMP-treated mice after the first immunization, with titers in the latter being about fivefold greater. Following the

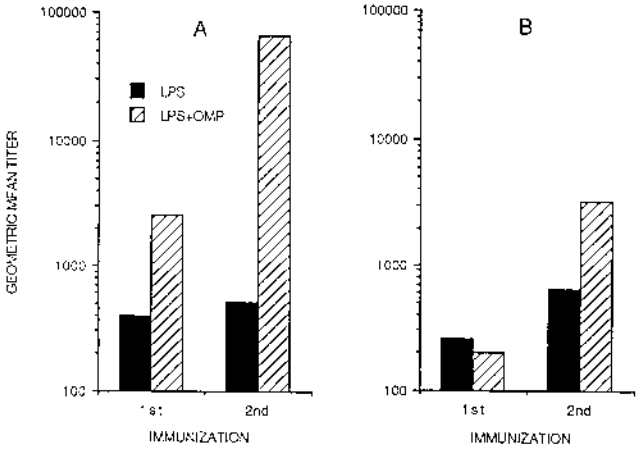


FIG. 5. Serum IgG1 (A) and IgG2a (B) subclass responses of mice treated with LPS or LPS-GBOMP to *B. melitensis* LPS after the first and second immunizations (sera from days 28 and 43, respectively). Each data point represents the geometric mean titer for three animals.

second immunization, IgG1 titers in LPS-GBOMP-treated mice increased about 30-fold, while titers in LPS-treated mice were essentially unchanged. IgG2a antibodies (Fig. 5B) were detected at about the same level in the two groups of mice after primary immunization. However, after the second immunization IgG2a titers in LPS-treated mice increased only 2-fold while in LPS-GBOMP-treated mice the titers increased about 15-fold.

## DISCUSSION

*Brucella* continues to pose health threats worldwide. In industrialized countries, livestock handlers and abattoir workers are the most at risk of infection, while in less developed parts of the world, consumption of unpasteurized dairy products from goats and cows facilitates the spread of disease to humans (28). Immune mechanisms which protect against *Brucella* are not well understood. Both antibody- and cell-mediated mechanisms probably play a role. Activation of the immune system by infection with a heterologous intracellular pathogen was shown to provide nonspecific protection against *B. abortus* infection in mice; this protection may be mediated by activated macrophages (16). In cattle, live attenuated rough strains (LPS deficient) which induced lymphoproliferative responses, but not LPS antibodies, were able to confer protection against infection and to lower the frequency of abortion (8). On the other hand, passively administered LPS monoclonal antibodies were shown to lower the bacterial burden (9) or prevent infection in mice (24) challenged with *B. abortus* by parenteral routes. The ability of local antibodies to protect against infection by a mucosal route has not been established.

Of the *Brucella* spp., smooth strains *B. melitensis*, *B. abortus*, and *B. suis* are virulent for humans, with *B. melitensis* causing the most severe disease. The LPSs of *B. abortus* and *B. melitensis* are both composed of repeating units of 4,6-dideoxy-4-formamido-α-D-mannopyranosyl residues. *B. abortus* LPS (A LPS) is a homopolymer of 1,2-linked units, while in *B. melitensis* LPS (M LPS) there are repeating pentamers of a single 1,3-linked and four 1,2-linked units (5). Thus, polyclonal antibody responses to *B. melitensis* LPS may recognize separate sets of epitopes associated with the different linkages. In our study, the serum LPS antibody responses of both mice and guinea pigs showed cross-reactivity with *B. abortus* LPS, suggesting that immunization with the LPS-GBOMP vaccine may have the potential to provide cross-protection. Cross-reactive antibodies recognizing *B. abortus* LPS were also detected in the lung lavages of both mice and guinea pigs (data not shown).

By complexing the LPS of *B. melitensis* with GBOMP, we have developed an intranasal vaccine capable of stimulating specific IgA and IgG antibodies in both humoral and mucosal compartments. The vesicular nature of the complex, demonstrated by electron microscopy, probably increases the adhesion of the vaccine to mucosal surfaces; this may in turn enhance uptake and processing of the LPS by antigen-presenting cells. The complex was immunogenic in both mouse and guinea pig models. While the guinea pigs immunized with LPS alone developed specific pulmonary antibodies of all three isotypes, the levels of these antibodies were significantly lower than those of animals given the complex vaccine. After a single immunization, the complex vaccine stimulated significantly higher levels of LPS-specific pulmonary IgA and serum IgG and IgA in mice than immunization with LPS alone. The high levels of serum antibodies elicited by the complex vaccine may provide a second line of defense against brucellae which have crossed the mucosal barrier. Immunologic memory was also stimulated, since it was shown that boosting with the complex vaccine in mice caused a typical secondary response to *B. melitensis* LPS. Mice treated with LPS alone developed a predominantly IgM response typically associated with T-independent antigens. It has been shown that polysaccharide antigens, normally T independent in mice, stimulate serum IgG when coupled to GBOMP and given as a parenteral vaccine (11). GBOMP has also been shown to have T helper cell mitogenic activity (14).

Our results suggest that the presence of the GBOMP may have induced a strong Th2-type IgG response to *B. melitensis* LPS that was absent in LPS-treated mice. This is consistent with the induction of high levels of IgA, which are also associated with Th2 activity. In addition, the Th1 response was also stimulated, since IgG2a antibody responses were enhanced in LPS-GBOMP-immunized mice. Studies under way in our laboratory will test whether the immune responses induced by the mucosal immunization strategy described in this paper will protect against mucosal challenge with *Brucella.*

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