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Immunoglobulin M and Immunoglobulin G Responses in BALB/c Mice to Conjugated Outer Membrane Extracts of Four *Salmonella* Serotypes†

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Outer membranes (OMs) of *Salmonella enteritidis*, *S. anatum*, *S. typhimurium*, and *S. infantis* were extracted and cross-linked with glutaraldehyde to form a large macromolecular antigen. The antigen consisted of OM proteins and lipopolysaccharide and was designated 4-OMP-LPS. Polyacrylamide gel electrophoresis of extracted OMs from each serotype revealed differences in protein profiles. *S. enteritidis* and *S. infantis* possessed a greater variety of proteins than did *S. anatum* and *S. typhimurium*. Immunizations with 4-OMP-LPS in phosphate-buffered saline (4-OMP-LPS-C) and 4-OMP-LPS emulsified with muramyl dipeptide in the oil phase of a hexadecane-water emulsion (4-OMP-LPS-MDP) revealed that BALB/c mice were capable of eliciting specific primary and secondary immunoglobulin M (IgM) and IgG responses. Both antigen preparations were capable of eliciting IgM and IgG specific for the cell surfaces of each live *Salmonella* serotype. Also, 4-OMP-LPS-MDP and 4-OMP-LPS-C were capable of evoking a substantial anamnestic response. Adsorption studies revealed that the combined serotypes had the antigenic capacity to adsorb up to 94% of the antibodies, but 4-OMP-LPS-MDP antibodies were more effectively adsorbed than were 4-OMP-LPS-C antibodies. Adsorption of pooled antiserum with heterologous bacteria yielded a variety of adsorption profiles.

Outer membranes (OMs) of several gram-negative bacteria are capable of eliciting humoral antibody responses (7, 8, 18, 21, 22, 28, 51). It has been found that outer membrane proteins (OMPs) prepared from *Escherichia coli* are potent B-cell mitogens capable of inducing a polyclonal antibody response (28). Additionally, the *E. coli* OMP preparation was mitogenic for lipopolysaccharide (LPS) nonresponder C3H/HeJ mice and could act as an adjuvant by enhancing antibody responses specific for sheep erythrocytes (28).

Karch and Nixdorff (18) demonstrated that an OMP isolated from *Proteus mirabilis* had the capacity to elicit antibody responses typical of thymus-dependent antigens. The immunoglobulin G (IgG) response could be increased if the OMP was complexed with either LPS or vesicles of phospholipids extracted from *P. mirabilis*. Conversely, OMP was capable of significantly increasing the humoral response to LPS by shifting the response from predominantly IgM to predominantly IgG.

Kuusi et al. (22), by using an OMP (porin) preparation extracted from a rough strain of *Salmonella typhimurium*, were successful in eliciting high antiporin and high anti-LPS titers in both mice and rabbits. Both porins and rabbit antiserum to porins provided significant protection in mice challenged with smooth *S. typhimurium*. When the rabbit antiporin serum was adsorbed with an LPS immunosorbent, the protective capacity of the serum did not change, indicating that antiporin antibodies were the protective components. After further purification of the porin preparation (21) to remove LPS and lipoproteins, it was found that the protective capacity of the antiserum against the purified porin preparation was lost. However, on reconstitution of the vaccine with homologous LPS, the protective capacity was restored.

Winter et al. (51) investigated immune responses in cattle vaccinated with whole cells, OMs, and OMPs prepared from *Brucella abortus*. These workers reported that OMP was capable of inducing significant antibody and delayed hypersensitivity responses. Recently, a purified OMP-F (porin) preparation isolated from *Pseudomonas aeruginosa* was shown to protect mice both actively and passively (7). Other studies have reported that mice infected with *P. aeruginosa* elicited antibodies specific for OMPs (8).

The observation that OMPs complexed with LPS enhanced the synthesis of IgG (18) suggested that it may be feasible to prepare a novel immunizing agent by cross-linking OM components. Such a preparation would have several advantages over antigens consisting of either single or unconjugated OM components. (i) It would be more economical; (ii) it would allow the construction of OM polyvalent vaccines containing either homologous or heterologous OM antigens; (iii) it would allow the utilization of many OM components that may have a greater enhancing value when cross-linked than if present as single components; (iv) it would present to the immune system surface antigens which may otherwise be hidden in the OM on the intact organism(s); and (v) such a complex molecule containing increased protein may be able to elicit both humoral and cell-mediated immune responses. It is for these reasons that we designed experiments to: (i) extract OMs of four prominent *Salmonella* serotypes representing four serologically different serogroups and compare their OMP profiles, (ii) utilize as many of these extractable OMP components (including a small amount of LPS) as possible and conjugate the OMPs with glutaraldehyde to form a cross-linked antigen, and (iii) examine the primary and secondary IgM and IgG responses specific for both the antigen and the live homologous organisms.

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MATERIALS AND METHODS

Bacterial strains and growth conditions for preparation of cell walls. *S. enteritidis* NCTC 5694 serogroup D was obtained from F. M. Collins (Trudeau Institute, Inc., Saranac Lake, N.Y.). *S. anatum* (83-4586) serogroup E_1 and *S. infantis* (83-4442) serogroup C_1 were obtained from B. O. Blackburn (National Veterinary Services Laboratories, Ames, Iowa). *S. typhimurium* ATCC 14028 serogroup B was obtained from the American Type Culture Collection (Rockville, Md.). All organisms were serotyped by the method of Ewing (5) and, after identification, were maintained on Trypticase soy agar (BBL Microbiology Systems, Cockeysville, Md.) supplemented with 5% sheep blood.

Large quantities of cells were obtained from spread plates of each organism on the same medium. After 18 h of incubation at 37°C in increased humidity, cells were removed from the plates, washed six times in 0.9% NaCl and twice with deionized water, and stored at −60°C.

Isolation and extraction of cell walls. OMs were extracted from each *Salmonella* serotype by a modification of the method of Nixdorff et al. (30). Frozen cells were thawed, mechanically deflagellated, and washed three times with deionized water and once with 0.02 M $NaHCO_3$. After washing, cells were suspended in 0.4% (wt/vol) 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate prepared by the procedure of Hjelmeland (10). Cells were broken by releasing the slurry from a French pressure cell (Travenol Laboratories, Inc., Savage, Md.) operated at an internal pressure of 1,406 kg/cm^2 while maintaining an approximate temperature of 4°C. The cell wall fraction was collected by centrifuging for 60 min at 30,000 × *g* at 4°C. The resulting cell wall pellet was washed three times with 0.02 M $NaHCO_3$ and three times with deionized water and then extracted for 60 min with five volumes of 90% (vol/vol) acetic acid at room temperature. The mixture was centrifuged for 15 min at 7,500 × *g* at 4°C, and the supernatant was dialyzed against deionized water at 4°C for 72 h. The OM material precipitating during dialysis was collected by centrifugation for 40 min at 15,000 × *g* at 4°C, and the pellet was dissolved in 1.0 M NaOH and neutralized with 0.1 M sodium acetate buffer (pH 4.5) containing 0.1 M NaCl. The solution was dialyzed against deionized water at 4°C for 48 h and centrifuged for 15 min at 7,500 × *g* at 4°C. The supernatant containing the OMP was lyophilized and stored at 4°C. Because it was the objective of this research to investigate the humoral immune responses of a conjugated fraction of OM containing a small amount of LPS, no further purification was performed.

Glutaraldehyde conjugation of OMPs. OMPs (500 mg) from each *Salmonella* serotype were combined and dissolved in 400 ml of 0.1 M phosphate-buffered saline (PBS; pH 8.3) and centrifuged for 15 min at 9,000 × *g* at room temperature. The supernatant was warmed to 37°C in a water bath, and glutaraldehyde (Aldrich Chemical Co., Inc., Milwaukee, Wis.) was added to a final concentration of 0.1% (37). Conjugation proceeded at 37°C for 60 min with occasional agitation, which resulted in a green-yellow solution. The solution was dialyzed against deionized water at 4°C for 24 h and lyophilized. The conjugate was rehydrated with deionized water, centrifuged for 20 min at 12,000 × *g* at 4°C, and washed with deionized water in an ultrafiltration cell (Amicon Corp., Danvers, Mass.) equipped with a YM30 membrane filter. After thorough washing, the solution was concentrated and lyophilized, and the final product (4-OMP-LPS) was stored under reduced pressure at −22°C.

Chemical analyses of 4-OMP-LPS. Total nitrogen content was estimated by the micro-Kjeldahl method of Kabat and Meyer (15) with bovine serum albumin as a standard. Protein content was determined by the Lowry method (24) with bovine serum albumin as a standard. 2-Keto-3-deoxyoctonate was estimated by the method of Karkhanis et al. (20) with 2-keto-3-deoxyoctonate (Sigma Chemical Co., St. Louis, Mo.) as a standard. Hexosamine was estimated as described by Nowotny (31). Samples were hydrolyzed in 3 N HCl for 4 h at 100°C, evaporated to dryness over KOH pellets under reduced pressure, suspended in deionized water, neutralized with 1.0 N NaOH, and analyzed with D-(+)-glucosamine hydrochloride (Sigma) as a standard.

SDS-PAGE of OMPs. OMPs from each *Salmonella* serotype were separated by discontinuous sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with the gel formulation of Lugtenberg et al. (26) in an electrophoretic apparatus described by Matsudaira and Burgess (27). Gel slabs were 67 mm in width, 80 mm in length, and 1.0 mm in depth, with separating and stacking gels containing 11 and 3% acrylamide, respectively. Protein molecular weight standards, expressed as molecular weight × 10^3 followed by the letter K (1), were bovine serum albumin (66K), ovalbumin (45K), and trypsinogen (24K) (Sigma). OMPs (15 μg) were applied to each well. Electrophoresis was carried out at a constant amperage of 9 mA per gel slab until the tracking dye was 5 mm from the bottom of the gel. Gels were stained for 4 h at room temperature in a solution containing 0.1% (wt/vol) Brilliant Blue R (Sigma), 50% absolute methanol, 10% acetic acid, and 40% deionized water. Gels were destained in a solution of 35% absolute methanol–7% acetic acid–58% deionized water until the desired clarity was obtained.

Densitometer scans of SDS-PAGE-separated OMPs. Separated OMPs from each *Salmonella* serotype were density scanned with a model 2520 gel scanner (Gilford Instrument Laboratories, Inc., Oberlin, Ohio). A Gilford model 252 photometer and a model DU spectrophotometer (Beckman Instruments, Inc., Fullerton, Calif.) were used as light source and wavelength control, respectively. Protein band densities were recorded on a Gilford model 6051 recorder. Electrophoretic mobilities (relative mobilities) of protein peaks were calculated (1). For identification of shared proteins, peaks possessing the same relative mobilities were assigned numbers.

Mice. Female inbred BALB/c mice (Harlan-Sprague-Dawley, Inc., Madison, Wis.) 6 weeks of age were used in all experiments. Mice were housed five to an Econofilter covered cage (Maryland Plastics, Inc., New York, N.Y.) with free access to food and water.

Preparation of antigen emulsions. Emulsions containing 2.0 mg of 4-OMP-LPS with 2.0 mg of muramyl dipeptide (MDP; Calbiochem-Behring, La Jolla, Calif.), 1.5% hexadecane (Sigma), 1.05% polyoxyethylene-sorbitan-mono-oleate (Tween 80; Sigma), and 0.45% sorbitan-mono-oleate (Emsorm 2500; Emery Industries, Mauldin, S.C.) were designated 4-OMP-LPS-MDP and were used for primary and secondary immunizations. Emulsions excluding 4-OMP-LPS were designated MDP-C and were used for inoculation of primary and secondary adjuvant control mice. Oil-water emulsions were prepared as described elsewhere (L. F. Woodard and R. L. Jasman, Vaccine, in press). MDP (2 mg) in 160 μl of absolute methanol was placed in a dry, sterile 15-ml glass-Teflon homogenizer. 4-OMP-LPS (2 mg) was transferred to homogenizers which were designated 4-OMP-LPS-MDP. After evaporation of methanol from all homoge-

nizers, 60 μl of hexadecane, 42 μl of Tween 80, and 18 μl of Emsorm 2500 were added. Mixtures were emulsified for 60 s with a motor-driven pestle operated at 1,500 rpm before addition of sterile 0.1 M PBS (pH 7.3) to achieve a final volume of 4.0 ml. Mixtures were homogenized twice for 2 min. Microscopic examination of antigen emulsions revealed uniform oil droplets with a shaded appearance (52).

Immunization of mice. For primary immunization experiments, 12 groups of five mice each were vaccinated subcutaneously (s.c.) in the nape of the neck with 0.2 ml of 4-OMP-LPS-MDP, 0.2 ml of MDP-C, or 100 μg of 4-OMP-LPS in 0.2 ml of PBS (4-OMP-LPS-C). On postinoculation days (PIDs) 7, 14, 21, and 28, mice were bled by cardiac puncture after anesthetization. Individual sera were collected and stored at −60°C. Secondary immunization experiments with mice and dosages as in primary experiments were carried out by administering the primary dose on day 0 and the secondary dose s.c. in the base of the tail on PID 20. Serum was collected, as previously described, on PIDs 25, 30, 35, and 40.

Determination of 4-OMP-LPS-specific IgM and IgG by ELISA. Immunoglobulin analyses were carried out by a microplate modification of the enzyme-linked immunosorbent assay (ELISA) technique described by Voller et al. (50). Briefly, polystyrene microtiter plates (no. 25860; Corning Glass Works, Corning, N.Y.) were coated by overnight evaporation of 10 μg of 4-OMP-LPS per 50 μl of deionized water per well. Wells were washed once with 0.2 M PBS (pH 7.3). To reduce nonspecific binding, we added 100 μl of a solution containing 0.1% gelatin (Sigma) in PBS to each well. After incubation at 37°C for 30 min, wells were washed once with PBS, and 100 μl of a serially diluted serum sample (diluted in PBS) was added to each well. After 1 h of incubation at 37°C, wells were washed three times with PBS, and 100 μl of specific anti-immunoglobulin-conjugated enzyme (peroxidase-conjugated goat anti-mouse IgG antibody or peroxidase-conjugated goat anti-mouse IgM antibody [Tago Inc., Burlingame, Calif.]) was added to appropriate wells. Anti-immunoglobulin-conjugated enzyme for IgM assay was diluted 1:700, and anti-immunoglobulin-conjugated enzyme for IgG assay was diluted 1:1,500. Both conjugates were diluted in PBS containing 0.05% gelatin. After 1 h of incubation at 37°C, wells were washed three times with PBS and twice with deionized water, and 100 μl of substrate solution was added to each well. Substrate solution consisted of 0.08% 5-aminosalicylic acid (pH 6.0) in deionized water with 0.05% H_2O_2 added before use. After 1 h of incubation at 37°C in the dark, the optical density of each well was determined with a microplate reader (no. EL 307; Bioteck, Burlington, Vt.). ELISA controls consisted of triplicate wells in which: (i) 4-OMP-LPS had not been dried to wells, (ii) PBS was added in place of serum, and (iii) PBS was added in place of anti-immunoglobulin-conjugated enzyme. Sera of untreated mice were included as negative serum controls. Test serum was analyzed by triplicate analyses of each serially diluted serum sample. Antibody titers were defined as the highest serum dilution giving an optical density above the statistical mean of negative serum controls.

Determination of IgM and IgG specific for live homologous *Salmonella* spp. Antibodies elicited by immunizations with 4-OMP-LPS-MDP and 4-OMP-LPS-C were analyzed for specificity to homologous live *Salmonella* spp. by ELISA as described by Ison et al. (13). Briefly, *S. enteritidis*, *S. anatum*, *S. typhimurium*, and *S. infantis* were grown on 5% blood agar plates for 18 h at 37°C in increased humidity. Each serotype was washed once with 0.2 M PBS (pH 7.3), suspended in PBS, and adjusted to an optical density of 0.5 at 700 nm.

Polystyrene microtiter plate (no. 25860; Corning) wells were filled with poly-L-lysine (Sigma) at 50 μg/ml in PBS and incubated at room temperature for 30 min. Wells were washed three times with PBS, and 100 μl of bacterial suspension was added to appropriate wells. After 30 min of incubation at room temperature, nonadherent bacteria were removed by aspiration, and wells were washed three times with PBS. Plate wells coated with live bacteria were filled with PBS containing 0.1 M glycine and stored in increased humidity at 4°C.

Before use, plates were allowed to reach room temperature, and wells were washed twice with PBS and filled with PBS containing 0.1% gelatin (Sigma) to reduce nonspecific binding. The ELISA procedure was then continued as described previously, except that anti-immunoglobulin-conjugated enzyme for IgM assay was diluted 1:500 and anti-immunoglobulin-conjugated enzyme for IgG assay was diluted 1:800.

Adsorption of antisera with homologous and heterologous bacteria. We prepared antisera for adsorption studies by immunizing mice s.c. in the nape of the neck with 0.2 ml of 4-OMP-LPS-MDP or 4-OMP-LPS-C on day 0 and administering a secondary dose s.c. in the base of the tail on PID 20. Antisera were collected, as previously described, on PID 30.

Homologous *Salmonella* strains used in these experiments were described above. Heterologous bacteria used are listed in Table 1. All bacteria were grown on 5% sheep blood agar plates for 18 h at 37°C. Cells were washed once with 0.2 M PBS (pH 7.3), suspended in PBS, and adjusted to McFarland nephelometer tube 9. Specified bacterial suspensions (500 μl each) were centrifuged for 10 min at 8,500 × *g*, and the supernatants (PBS) were removed. Antisera (80 μl) from either 4-OMP-LPS-MDP- or 4-OMP-LPS-C-immunized mice were mixed with bacterial cells and incubated in a 37°C water bath for 40 min. After incubation, tubes were sealed and refrigerated for 3 h. Cells were pelleted, and serum was sterilized by centrifugation through 0.2-μm (pore size) cellulose acetate filters (Centrex DF122/1; Schleicher & Schuell, Inc., Keene, N.H.). After overnight storage at 4°C, sera were analyzed for 4-OMP-LPS-specific IgM and IgG by ELISA as previously described.

TABLE 1. Heterologous bacteria used in adsorption of antisera

Bacterium	Source[a]
Arizona hinshawii	University of Idaho reference culture
Enterobacter aerogenes	ATCC 13048
Escherichia coli	ATCC 25922
Klebsiella pneumoniae	ATCC 13883
Proteus mirabilis	University of Idaho reference culture
Pseudomonas aeruginosa	ATCC 27853
Serratia marcescens	ATCC 8100
Yersinia enterocolitica	University of Idaho reference culture
Staphylococcus epidermidis	ATCC 12228

[a] ATCC, American Type Culture Collection.

Statistical analyses. Antibody titers, both IgM and IgG, determined from each mouse on each bleed day were converted to $\log_2 + 1$ and compared statistically. Either the Fisher exact test (46) or the Student *t* test (34) was used to calculate two-tailed *P* values for differences in titers. Statistical analyses were not performed on adsorption experi-

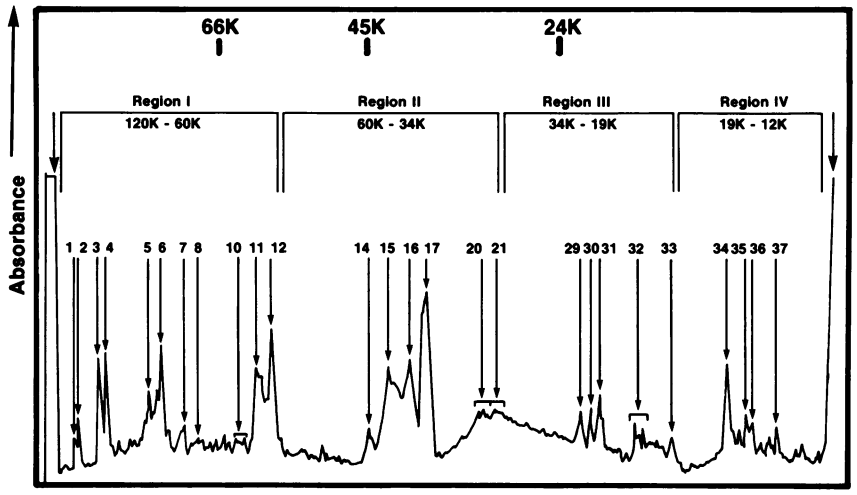

FIG. 1. Densitometer scan of SDS-PAGE-separated OMPs obtained from *S. enteritidis*. Dominant protein regions are indicated, and major protein peaks are numbered. Positions of protein molecular weight standards are indicated, and arrows at the left and right sides indicate the top of the separating gel and the position of the tracking dye, respectively. Absorbance was measured at 590 nm.

ments owing to inadequate quantities of antisera. Data were either significant ($P < 0.05$), highly significant ($P < 0.01$), or not significant ($P > 0.05$).

RESULTS

Comparison of OMPs from *Salmonella* serotypes by SDS-PAGE. OMPs from each *Salmonella* serotype were solubilized in 0.0625 M Tris hydrochloride (pH 6.8), separated by SDS-PAGE, and examined by densitometer scanning (Fig. 1 through 4). Each serotype possessed OMPs with molecular weights ranging from 12K to 120K, which were arbitrarily classified into four electrophoretic regions.

Region I in each serotype provided a contrasting profile on the uniqueness that characterized the OMPs of these organisms. *S. enteritidis* displayed the greatest variety and quantity of OMPs. Proteins 4, 6, 10, and 12 were shared among *S. enteritidis*, *S. infantis*, and *S. typhimurium*, whereas *S. anatum* was largely deficient of OMPs. Only protein 4 was shared by all serotypes.

Region II in each serotype consisted of multiple protein peaks and possessed the greatest amount of protein. As indicated, proteins 14 through 17 migrated closely and accounted for the majority of proteins for each serotype. *S. typhimurium* did not produce these proteins in quantity as did the other serotypes. In addition, *S. typhimurium* OMs were deficient in proteins 19 through 24.

Region III was a protein-rich region for each serotype, with proteins 30 and 31 commonly shared. These two proteins were quantitatively the most significant proteins for each serotype except *S. typhimurium*, which produced large quantities of protein 26 and other major proteins that were not apparent in the OMPs of the other serotypes.

Low-molecular-weight proteins occurring in region IV were numerous and in high quantity in *S. enteritidis*, which shared proteins 34 and 36 with *S. anatum*. *S. infantis* and *S. typhimurium* did not possess any major proteins in this region.

Chemical analyses. Chemical analyses revealed that 4-OMP-LPS contained 11.21% nitrogen, 71.10% protein, 0.48% 2-keto-3-deoxyoctonate, and 3.63% hydrolyzable hexosamine.

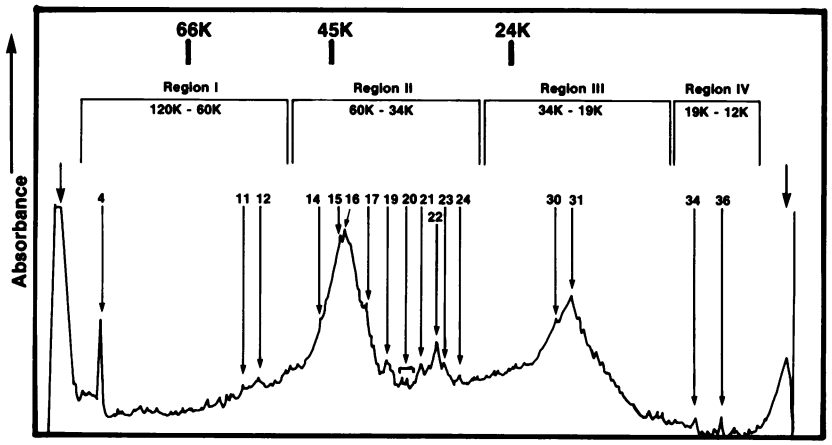

FIG. 2. Densitometer scan of SDS-PAGE-separated OMPs obtained from *S. anatum*. Dominant protein regions are indicated, and major protein peaks are numbered. Positions of protein molecular weight standards are indicated, and arrows at the left and right sides indicate the top of the separating gel and the position of the tracking dye, respectively. Absorbance was measured at 590 nm.

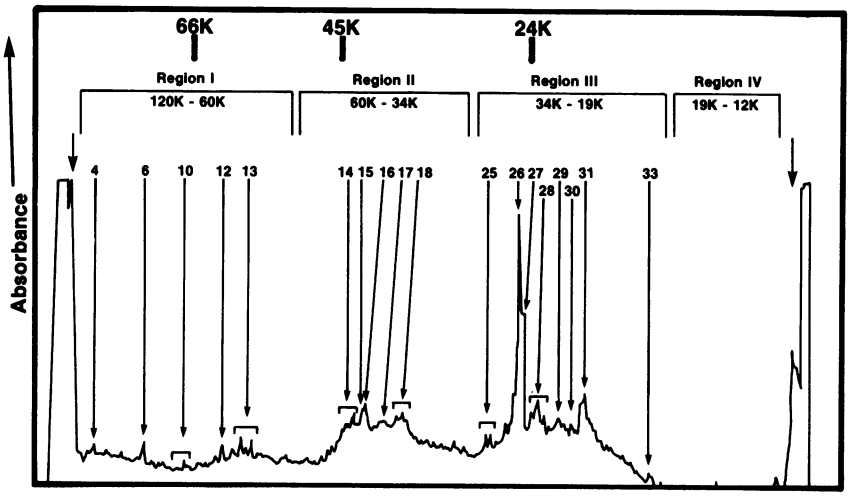

FIG. 3. Densitometer scan of SDS-PAGE-separated OMPs obtained from *S. typhimurium*. Dominant protein regions are indicated, and major protein peaks are numbered. Positions of protein molecular weight standards are indicated, and arrows at the left and right sides indicate the top of the separating gel and the position of the tracking dye, respectively. Absorbance was measured at 590 nm.

Primary IgM and IgG responses to 4-OMP-LPS. Mice were administered primary doses of immunizing agents on day 0 and monitored on PIDs 7, 14, 21, and 28 for 4-OMP-LPS-specific IgM and IgG. Mice immunized with 4-OMP-LPS-C or 4-OMP-LPS-MDP showed no significant differences ($P > 0.05$) between treatment groups or days within a treatment group, indicating that the level of specific IgM remained virtually constant from PIDs 7 through 28 and that the addition of MDP did not enhance the IgM response (Fig. 5). In addition, MDP-C elicited 4-OMP-LPS-specific IgM. This response was quite erratic and did not vary significantly between titers on different days ($P > 0.05$). Mice immunized with either 4-OMP-LPS-MDP or 4-OMP-LPS-C achieved higher titers than did MDP-C or normal control mice ($P < 0.05$ or 0.01).

Steady increases in 4-OMP-LPS-specific IgG were produced by both 4-OMP-LPS-MDP- and 4-OMP-LPS-C-immunized mice over the 28-day experimental period (Fig. 6). Immunizations with 4-OMP-LPS-MDP elicited higher titers on PIDs 14, 21, and 28 in comparison with immunizations with 4-OMP-LPS-C ($P < 0.05$ and $P < 0.01$). IgG titers from mice receiving 4-OMP-LPS-MDP were greater ($P < 0.01$) than those of controls on PIDs 7 through 28; however, titers from mice immunized with 4-OMP-LPS-C were significantly greater ($P < 0.01$) than those of controls only on PID 14.

Secondary IgM and IgG responses to 4-OMP-LPS. Mice were administered primary doses of immunizing agents on day 0, immunized with secondary doses on PID 20, and monitored for 4-OMP-LPS-specific IgM and IgG on PIDs 25, 30, 35, and 40. Immunizations with 4-OMP-LPS-MDP produced the highest secondary IgM titers on PID 25, but titers steadily decreased over the next 15 days (Fig. 5). Primary IgM titers elicited by either 4-OMP-LPS-C or 4-OMP-LPS-MDP were increased ($P < 0.01$) by secondary immunizations. However, when titers of 4-OMP-LPS-C-immunized mice were compared with titers of 4-OMP-LPS-MDP-immunized mice, differences in the levels of IgM could not be detected for PIDs 25 and 30 ($P > 0.05$). Differences in titers became apparent, however, on PIDs 35 and 40 ($P < 0.05$).

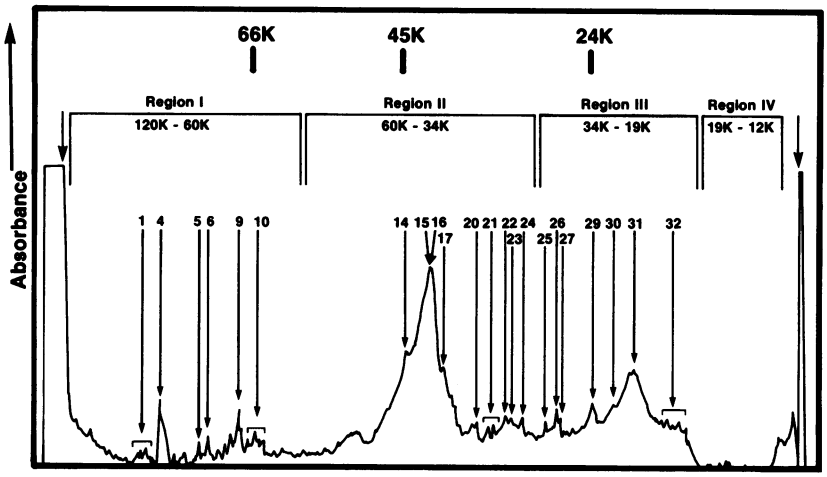

FIG. 4. Densitometer scan of SDS-PAGE-separated OMPs obtained from *S. infantis*. Dominant protein regions are indicated, and major protein peaks are numbered. Positions of protein molecular weight standards are indicated, and arrows at the left and right sides indicate the top of the separating gel and the position of the tracking dye, respectively. Absorbance was measured at 590 nm.

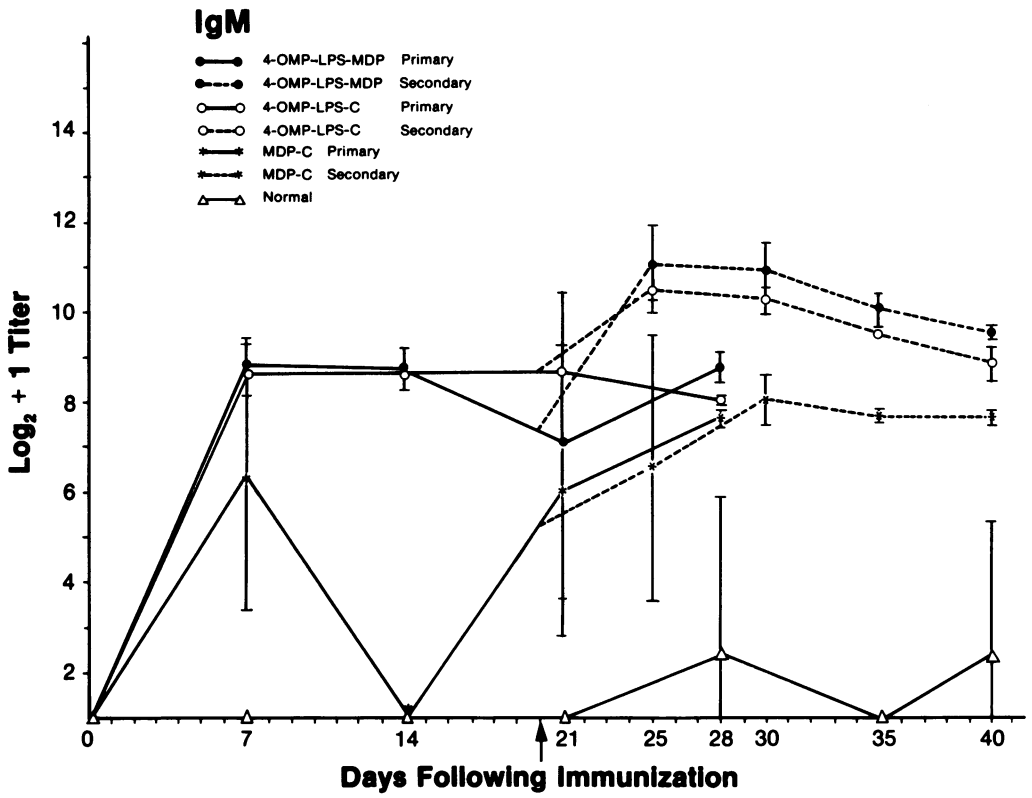

FIG. 5. ELISA titers of 4-OMP-LPS-specific IgM primary and secondary antibody responses. Data are expressed as the mean titers of five mice ± the standard deviation. Secondary immunizations given on day 20 are indicated by an arrow.

MDP-C-immunized mice did not respond to secondary immunizations ($P > 0.05$). Mice receiving either 4-OMP-LPS-C or 4-OMP-LPS-MDP elicited higher IgM titers than did MDP-C or normal control mice ($P < 0.01$).

IgG titers (Fig. 6) were markedly increased ($P < 0.01$) after secondary immunizations with either 4-OMP-LPS-MDP or 4-OMP-LPS-C. Mice immunized with 4-OMP-LPS-MDP responded with higher IgG titers on PIDs 25, 30, and 40 than did 4-OMP-LPS-C-immunized mice ($P < 0.05$). Both treatment groups responded with significantly greater titers than did control mice ($P < 0.01$).

Specificity of primary IgM and IgG toward live homologous *Salmonella* spp. Mice were administered primary doses of immunizing agents on day 0 and monitored on PIDs 7, 14, 21, and 28 for *S. enteritidis*-, *S. anatum*-, *S. typhimurium*-, and *S. infantis*-specific IgM and IgG. Immunizations with either 4-OMP-LPS-MDP or 4-OMP-LPS-C elicited IgM and IgG directed specifically toward the homologous live organisms from which the OMs were extracted (Fig. 7). Primary immunizations with both 4-OMP-LPS-MDP and 4-OMP-LPS-C induced high levels of IgM specific toward *S. enteritidis* and *S. infantis* (Fig. 7A). In contrast, IgM responses toward *S. anatum* were comparatively lower. Similarly, immunizations with 4-OMP-LPS-MDP failed to induce *S. typhimurium*-specificIgM, whereas immunizations with 4-OMP-LPS-C elicited weak responses only on PIDs 7 and 14.

Primary immunizations with 4-OMP-LPS-MDP were capable of evoking a specific IgG response toward *S. infantis*, *S. enteritidis*, and *S. anatum* throughout the 28-day experimental period, whereas the IgG response toward *S. typhimurium* was observed only on PIDs 21 and 28 (Fig. 7B). Although immunizations with 4-OMP-LPS-C did not effect a specific IgG response toward *S. typhimurium*, specific IgG responses were observed for *S. infantis*, *S. anatum*, and *S. enteritidis*. Primary immunizations with 4-OMP-LPS-MDP induced higher IgG titers than did immunizations with 4-OMP-LPS-C ($P < 0.05$ and $P < 0.01$).

Specificity of secondary IgM and IgG toward live homologous *Salmonella* spp. Mice were administered primary doses of immunizing agents on day 0, immunized with secondary doses on PID 20, and monitored on PIDs 25, 30, 35, and 40 for *S. enteritidis*-, *S. anatum*-, *S. typhimurium*-, and *S. infantis*-specific IgM and IgG. Secondary immunizations with either 4-OMP-LPS-MDP or 4-OMP-LPS-C had the capacity to stimulate high IgM titers toward *S. enteritidis*, *S. anatum*, and *S. infantis*, although 4-OMP-LPS-MDP-immunized mice responded with statistically higher levels ($P < 0.05$ and $P < 0.01$) (Fig. 8A). Secondary IgM titers toward the four serotypes were significantly increased above primary IgM levels by immunizations with 4-OMP-LPS-MDP ($P < 0.05$), whereas secondary immunizations with 4-OMP-LPS-C did not increase IgM titers ($P > 0.05$). *S. typhimurium* IgM titers were lower in comparison with other serotypes ($P < 0.05$). However, immunizations with 4-OMP-LPS-MDP generally elicited higher *S. typhimurium* IgM levels than did immunizations with 4-OMP-LPS-C ($P < 0.05$).

Elevated secondary IgG titers toward all serotypes were observed after secondary immunizations with both 4-OMP-LPS-MDP and 4-OMP-LPS-C (Fig. 8B). IgG titers toward all serotypes in the secondary response were greater than in primary responses after immunizations with 4-OMP-LPS-

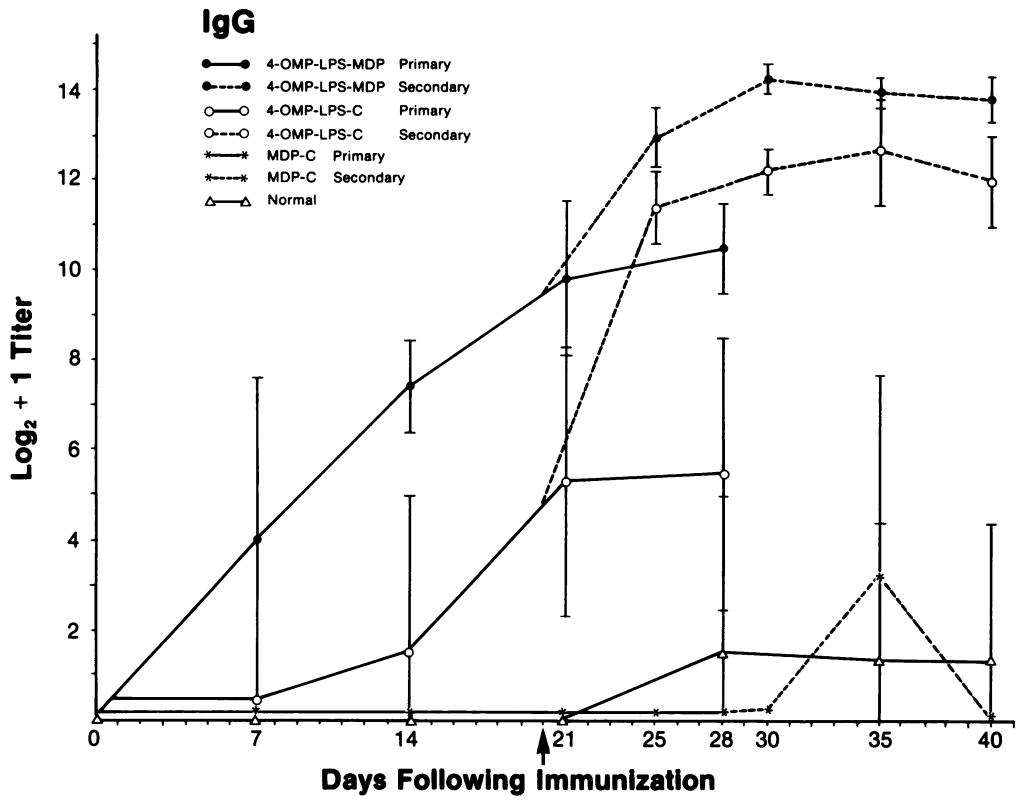

FIG. 6. ELISA titers of 4-OMP-LPS-specific IgG primary and secondary antibody responses. Data are expressed as the mean titers of five mice ± the standard deviation. Secondary immunizations given on day 20 are indicated by an arrow.

MDP ($P < 0.01$). In contrast, mice immunized with 4-OMP-LPS-C had higher IgG responses to only *S. enteritidis* and *S. infantis* ($P < 0.01$). However, 4-OMP-LPS-MDP had the capacity to elicit greater secondary IgG responses toward all serotypes than did 4-OMP-LPS-C ($P < 0.05$ and $P < 0.01$). When comparing responses toward all serotypes, it should be noted that immunizations with either 4-OMP-LPS-MDP or 4-OMP-LPS-C induced lower levels of specific IgM and IgG directed toward *S. typhimurium*. Mice receiving both primary and secondary immunizations of MDP-C, and normal mice, produced no detectable IgM or IgG specific toward any of the four live homologous serotypes.

Reductions of 4-OMP-LPS-specific IgM and IgG titers by adsorption with homologous and heterologous bacteria. 4-OMP-LPS-specific IgM and IgG titers were reduced by adsorption of antisera with homologous *Salmonella* spp. and heterologous bacteria (Table 2). Combined homologous *Salmonella* spp. were highly effective in adsorbing both IgM and IgG from both 4-OMP-LPS-MDP and 4-OMP-LPS-C antisera. Adsorptions with both individual and combined homologous *Salmonella* spp. resulted in greater reductions of specific IgM and IgG from 4-OMP-LPS-MDP antisera than from 4-OMP-LPS-C antisera.

With the exceptions of *P. mirabilis* and *Staphylococcus epidermidis*, 4-OMP-LPS-C IgG was not reduced by any of the heterologous bacteria, whereas 4-OMP-LPS-MDP IgG was reduced by all of the heterologous bacteria except *Yersinia enterocolitica*. The abilities of heterologous bacteria to reduce the IgM titers of both antisera were comparable, although *Arizona hinshawii*, *Enterobacter aerogenes*, *Serratia marcescens*, and *S. epidermidis* reduced 4-OMP-LPS-MDP IgM more effectively than 4-OMP-LPS-C IgM.

DISCUSSION

Results described in this study provide substantial evidence that conjugated *Salmonella* OM extracts have the capacity to elicit considerable IgM and IgG responses in BALB/c mice. These responses were specific toward the live homologous *Salmonella* serotypes from which the OMs had been extracted.

Within the OM complex, certain types of proteins were shared by all four serotypes; each serotype could be characterized as possessing a unique complement of proteins in regions I and IV. Similar protein constituents in these electrophoretic regions have been reported for other members of the *Enterobacteriaceae* (12). It is worth noting, however, that possible differences in OMP profiles may be due to either strain or serotype differences.

Region II was primarily dominated by four major proteins, which appeared to be analogous to the 33K, 34K, 35K, and 36K OM complex described by Ames et al. (2) in *S. typhimurium* and identified by other investigators (16, 25, 26, 32, 33, 49) in a variety of gram-negative bacteria. Aside from minor quantitative differences, it appears that this complex of proteins can be readily extracted, either fully or partially, by several extraction procedures, whereas other proteins are preferentially extracted (4). In contrast to the other three serotypes, fewer proteins were found in region II from extracts of *S. typhimurium*. Three possible explanations for this finding are suggested. (i) *S. typhimurium* does not normally produce large amounts of these major proteins. (ii) The proteins of *S. typhimurium* display different chemical and physical properties and are not extractable by the acetic acid procedure. (iii) The fractionation process could have

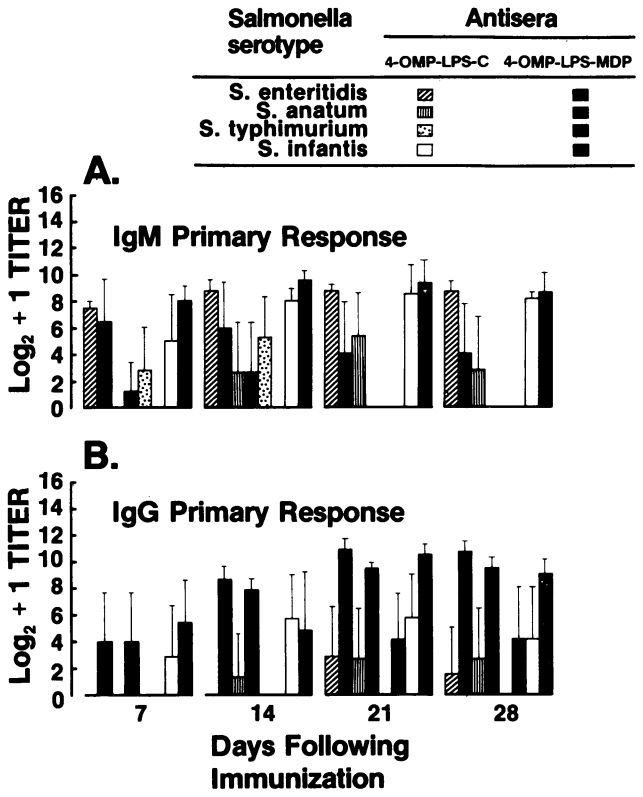

FIG. 7. ELISA titers of IgM and IgG specific toward live homologous *Salmonella* spp. elicited by primary immunizations with 4-OMP-LPS-C and 4-OMP-LPS-MDP. Data are expressed as the mean titers of five mice ± the standard deviation.

depolymerized the proteins into smaller subunits, which were lost during ultrafiltration.

Compared with the other three *Salmonella* serotypes, *S. typhimurium* displayed an abundance of protein 26 in region III. We were unable to correlate this result with the findings of other workers (29, 35, 48) who used different extraction procedures. Whether the production of this protein was enhanced by cultivating the organism on blood agar or whether it was an artifact produced during fractionation is unknown. In comparison, proteins 30 and 31 (which were present in all four serotypes) may be analogous to protein III described by Garten et al. (6) and Hofstra and Dankert (12).

It is interesting to observe that *S. typhimurium* is not endowed with the same array of OMPs as seen in the other three serotypes investigated in this study and that 4-OMP-LPS preparations elicited weak *S. typhimurium* responses in contrast to the other three *Salmonella* serotypes. Although there has been no evidence presented here which explains the biological manifestations of these differences, questions can be posed as to whether these differences are linked to: (i) its virulence, (ii) its inability to induce a protective immune response, or (iii) the prevalence of this serotype in clinical salmonellosis in both humans and animals.

Conjugation of the extracted OM components with glutaraldehyde formed a large cross-linked antigen (4-OMP-LPS) possessing a wide variety of OMPs and LPSs obtained from four *Salmonella* serotypes. These serotypes represent four serologically different *Salmonella* serogroups (B, C_1, D, and E_1) (5), which are frequently isolated from livestock. The present study provides evidence suggesting that different *Salmonella* serotypes may possess different and shared OMPs. We wanted to utilize as many extractable OMPs from these different serotypes as possible and, by cross-linking the OMPs, produce the corresponding serotype-specific immunogen. When 4-OMP-LPS was dissolved in PBS (4-OMP-LPS-C) or emulsified with MDP (4-OMP-LPS-MDP), heightened primary and secondary humoral immune responses were observed. In addition to 4-OMP-LPS-specific responses elicited from immunizations with 4-OMP-LPS-MDP and 4-OMP-LPS-C, antibody responses toward the live organisms from which the OMs were extracted were monitored. Considerable primary and secondary responses were elicited toward *S. enteritidis* and *S. infantis*, whereas *S. anatum* responses were somewhat lower, and responses toward *S. typhimurium* were usually lower than those toward the other three serotypes. We suggest that substantial humoral responses specific for *S. enteritidis* and *S. infantis* were elicited by quantitatively more extracted peptides in comparison with fewer peptides extracted from *S. anatum* and *S. typhimurium*. Adsorption experiments conducted with individual homologous *Salmonella* serotypes provided additional evidence that this may be the case. Reductions of IgM and IgG by *S. typhimurium* were considerably less in comparison with adsorptions by other *Salmonella* serotypes. It is also possible that OMPs extracted from *S. typhimurium* were less antigenic or were not cross-linked during the conjugation procedure.

In comparing immune responses elicited from immuniza-

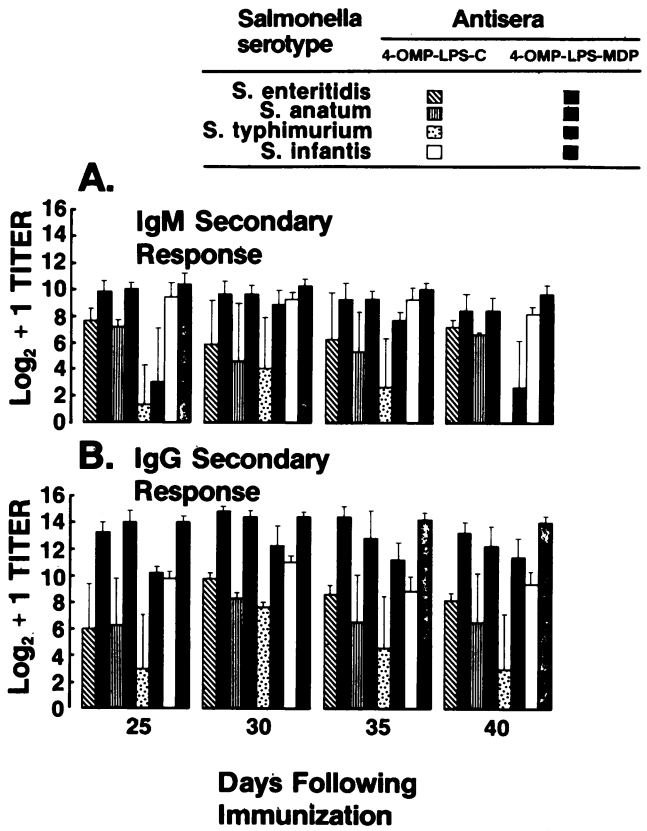

FIG. 8. ELISA titers of IgM and IgG specific toward live homologous *Salmonella* spp. elicited by secondary immunizations with 4-OMP-LPS-C and 4-OMP-LPS-MDP. Data are expressed as the mean titers of five mice ± the standard deviation.

TABLE 2. Reduction of 4-OMP-LPS-specific antibody titers after adsorption by homologous and heterologous bacteria

Organism added to immune sera	% Reduction of antibody titers of:			
	4-OMP-LPS-MDP antisera		4-OMP-LPS-C antisera	
	IgM	IgG	IgM	IgG
Control (no organism added)	0	0	0	0
Salmonella enteritidis	87.5	87.5	50.0	50.0
Salmonella anatum	75.0	87.5	50.0	50.0
Salmonella typhimurium	50.0	75.0	50.0	50.0
Salmonella infantis	87.5	87.5	50.0	75.0
Combined homologous *Salmonella* spp.[a]	93.75	93.75	75.0	87.5
Arizona hinshawii	75.0	75.0	50.0	0
Enterobacter aerogenes	75.0	50.0	50.0	0
Escherichia coli	50.0	50.0	50.0	0
Klebsiella pneumoniae	50.0	50.0	50.0	0
Proteus mirabilis	50.0	50.0	50.0	50.0
Pseudomonas aeruginosa	50.0	50.0	50.0	0
Serratia marcescens	87.5	87.5	0	0
Yersinia enterocolitica	50.0	0	ND[b]	ND[b]
Staphylococcus epidermidis	75.0	50.0	50.0	50.0

[a] Equal amounts of cells of each of the above homologous *Salmonella* spp. incubated in one tube with antiserum.
[b] ND, Not determined.

tions with 4-OMP-LPS-C and 4-OMP-LPS-MDP, it is interesting to observe that emulsions of MDP, oil, and 4-OMP-LPS gave only slightly higher titers than 4-OMP-LPS in PBS. Although the adjuvanticity of MDP was expected to enhance humoral responses, the inherent antigenicity of 4-OMP-LPS proved to be considerable, thus giving evidence that OM preparations may contain all necessary ingredients for satisfactory immune enhancement. Specific secondary IgG responses toward 4-OMP-LPS and individual *Salmonella* serotypes by 4-OMP-LPS-C suggest that IgG memory was probably induced. Previous studies in our laboratory (9, 11) and in others (17, 40, 43) showed that protein attached to LPS appeared to have changed the functional role of LPS from B-cell to T-cell dependency. In addition, data were presented which suggested that immunological memory, a T-cell-dependent function (43), was induced by an LPS-protein complex. In contrast to these findings, OMPs obtained from *E. coli* have been shown to induce polyclonal activation of murine lymphocytes but were unable to stimulate nylon-wool-purified T-cells or thymocytes (28). Although immunizations with 4-OMP-LPS-C elicited substantial titers, adsorption studies comparing 4-OMP-LPS-C and 4-OMP-LPS-MDP revealed that the latter antiserum was adsorbed more effectively by individual or combined homologous *Salmonella* spp. and heterologous bacteria. In addition, 4-OMP-LPS-C IgM levels were weakly reduced by heterologous bacteria, and IgG was generally not reduced, whereas the opposite was true for 4-OMP-LPS-MDP IgM and IgG. From these findings, we suggest that the addition of MDP promoted increased immune cell recognition and antibody syntheses specific for OMPs present in lesser amounts. This specificity led to increased homologous and heterologous adsorptions of 4-OMP-LPS-MDP antiserum by bacterial cell surfaces, whereas 4-OMP-LPS-C IgM adsorptions appeared uniform. To our knowledge, there have been no published reports that specifically describe the immune responses to conjugated OMPs of gram-negative bacteria. However, relevant to these studies are a number of reports describing the immune responses to a variety of OM preparations (3, 7, 8, 17, 19, 21, 22, 47, 51). These reports support our findings to the extent that OMPs are capable of stimulating a humoral immune response.

BALB/c mice have been shown to be genetically poor responders to *Salmonella* antigens (38, 39, 41, 42), but they do respond to bovine serum albumin in the presence of MDP (45). The present study provides evidence that 4-OMP-LPS, an antigen prepared from the OMs of four *Salmonella* serotypes, is capable of stimulating BALB/c mice to produce 4-OMP-LPS-specific IgM and IgG antibodies either by itself or in the presence of MDP.

It appears that LPS is required in salmonella-derived immunizing preparations for the attainment of protection, although low levels of LPS are not sufficient to elicit anti-LPS (21). Chemical analyses of 4-OMP-LPS revealed that small amounts of LPS were present, as indicated by the markers 2-keto-3-deoxyoctonate and hexosamine. Although anti-LPS titers were not determined, high anti-LPS levels were probably not involved, because the amount of LPS in 4-OMP-LPS was low and immunopotentiation was achieved, whereas large amounts of LPS cause immunosuppression (36).

Another point of interest in these studies is the observation that the adjuvant control (MDP-C) elicited small amounts of 4-OMP-LPS-specific IgM after both primary and secondary immunizations. Perhaps MDP-C activated the syntheses of IgM antibodies directed against intestinal gram-negative surface antigens which cross-react with *Salmonella* antigens. Since only IgM was elicited, and because IgM is predominantly produced in response to O-antigens (LPS) (14, 23, 44), this explanation seems plausible.

Current research in our laboratory is being conducted to determine which of the many OM components of the 4-OMP-LPS conjugated complex contributed to the humoral responses observed in these studies.

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